AN ABSTRACT OF THE DISSERTATION OF

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Joseph W. Spatafora

The inoperculate discomycete order Helotiales (Ascomycota) is a diverse and complex group of fungi which include lichens, earth tongues, and endophytes. Cladistic analyses of multiple loci were used to test and construct phylogenetic hypotheses and to examine the evolution of various life history strategies adopted by these fungi. Several prominent patterns of convergent evolution were revealed. Within the lichenized Helotiales, nuclear SSU and LSU rDNA data inferred two distinct lineages (i.e., Baeomycetaceae and Icmadophilaceae) representing potential independent gains of the lichen symbiosis. The common "baeomycetoid" morphology of these fungi, was shown to be the result of convergent evolution between these two distinct lineages. The correspondence of phylogenetic hypotheses from independent, multiple loci also presented reciprocal corroboration for convergent evolution of sporocarp morphology among the earth tongues. At least four independent lineages which possess the earth tongue fruitbody morphology were inferred from the three independent data sets of SSU

rDNA, combined SSU and LSU rDNA, and RPB2. The close phylogenetic relationship between the earth tongues, *Cudonia* and *Spathularia* (Helotiales) and some endophytes represented by members of the Rhytismatales was particularly provocative due to vast differences in sporocarp morphology, but was nonetheless well-supported and corroborated with these forms of independent molecular phylogenetic evidence. Phylogenetic hypotheses constructed from analyses of multiple loci inferred that the Helotiales are polyphyletic, a finding not consistent with the character of inoperculate asci being a synapomorphy for this group. Rather, polarization of this ascus character suggested that inoperculate asci may be symplesiomorphic for many of the euascomycetes. Other life history strategies were also examined within the lichenized Helotiales including the loss of sexual reproduction. Analyses of nuclear SSU and LSU rDNA prompted a proposal to emend the Icmadophilaceae to include the nonsexual genera *Siphula* and *Thamnolia* which were previously classified in the Lecanorales. Other revised classifications were proposed. The Geoglossaceae are redefined to include *Geoglossum, Sarcoleotia,* and *Trichoglossum*, but to exclude *Microglossum, Cudonia,* and *Spathularia*. The effect of fungal intragenic SPV on maximum parsimony was also examined.

Lichens, Earth Tongues, and Endophytes: Evolutionary Patterns Inferred from Phylogenetic Analyses of Multiple Loci

by

Jamie L. Platt

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Jamie L. Platt, Author

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CONTRIBUTION OF AUTHORS

Chapter four was co-authored with Francisco J. Camacho and Joseph W. Spatafora. Francisco Camacho made major contributions regarding data analyses, taxon sampling, and study design. All data collection was done in the laboratory of Joseph Spatafora, who also provided the funding for this study from his discretionary funds. Joseph Spatafora also provided expertise in phylogenetic analyses and cladistic theory.

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This dissertation is dedicated to the loving memory of my father who always nurtured my inquisitive nature.

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CHAPTER 1

Introduction and Literature Review

1.1 Abstract

The Helotiales is the only fungal order comprised of lichens, earth tongues, and endophytes. Although convergent evolution has resulted in lichen and endophyte symbioses, as well as earth tongue-like morphologies in other fungal lineages, current classification schemes do not recognize these evolutionary trends as occurring within a single order outside of the Helotiales. This group therefore represents the ideal assemblage of taxa with which to examine the evolution of some common pathways in fungal adaptations. The objective of this study is to construct sequence-based phylogenies for the Helotiales and infer evolutionary patterns of the inoperculate discomycetes. Cladistic analyses of nuclear rDNA sequence data will form the basis for reconstructing a more natural classification of the Helotiales and examining the monophyly of the order. In addition, a protein-coding nuclear locus (RPB2) will be used to construct phylogenetic hypotheses which can then be compared to the rDNA-based phylogenies. Congruent phylogenetic patterns between these two sources of independent data can then be interpreted to constitute reciprocal corroboration of accurate

phylogenetic evidence. The substantial taxon and character sampling of this study is novel to higher level ascomycete systematics and will serve as a benchmark of this field.

1.2 Introduction

The Fungi, sometimes known as the "Fifth Kingdom", are a diverse group of eukaryotic organisms which have adopted a vast range of life strategies and are found in almost every conceivable ecological niche. One of the most successful life strategies adopted by fungi is the establishment of symbiotic relationships. In fact, the successful colonization of the terrestrial environment by "plants" is hypothesized to be a consequence of an evolutionary shift to symbiosis between an autotrophic green alga and a heterotrophic fungus (Pirozynski and Malloch, 1975). Numerous examples of symbioses are exhibited by fungi; some of the best known are mycorrhizae, pathogens, endophytes, and lichens. Both phyla Basidiomycota and Ascomycota include lineages of fungi which form the symbioses types mentioned above, highlighting the predominance of patterns of convergent evolution within Fungi. However, within the ascomycetes, only a single order, the Helotiales, is comprised of mycorrhizae, pathogens, endophytes, and lichens. This group of inoperculate discomycetes therefore presents an excellent model system for examining evolutionary shifts to symbioses and various other life strategies. Furthermore, the distribution and diversity of life strategies suggests that if this order is monophyletic it has undergone a significant radiation event subsequent to the primary ascomycete radiation. A more recent radiation event, such as this, within the ascomycetes could lead to interesting hypotheses about fungal evolution.

The evolution of the lichen symbioses plays a central role in this study. The Helotiales presents the ideal background for polarizing the character of lichenization because the order is primarily non-lichenized. This implies that the lichen symbiosis is a relatively recent event within this lineage and therefore, lichenization events should be present near the middle or tips of the branches of inferred phylogenetic trees. In working towards obtaining phylogenetic evidence for the hypothesis that the lichen symbiosis has evolved within the Helotiales, it became apparent that I must first address several other important questions.

In 1993 two lichenized families, the Baeomycetaceae and Icmadophilaceae were reclassified in the order Helotiales (Rambold et al., 1993). Both of these families are comprised of lichenized taxa which exhibit a "baeomycetoid" morphology, i.e., shortly stipitate convex apothecia clustered on a thallus. Because most lichenized discomycetes are classified in the order Lecanorales, often simply by default, this ordinal reclassification was notable and gained much attention among lichen systematists. In fact, this reclassification based on ascus morphology was an important first step toward integrating lichenized and nonlichenized taxa in fungal classification schemes. In addition to these lichen families being placed within this primarily non-lichenized order, the genus *Dibaeis* was reintroduced (Gierl and Kalb, 1993) and moved from the Baeomycetaceae to the Icmadophilaceae (Rambold et al., 1993). This reclassification spawned a systematic debate which was brewing at the outset of this study. Thus, my first objective is to test the hypothesis that *Dibaeis* is best accommodated in the Icmadophilaceae and is indeed a distinct genus from *Baeomyces* of the Baeomycetaceae. In chapter 2, I address this taxonomic question and examine the evolutionary histories of

these two families of "baeomycetoid" lichens using cladistic analyses of nSSU and nLSU rDNA.

A second question arising indirectly from examining the evolution of baeomycetoid lichens was the evolution of nonsexual lichenized taxa. Initially, in designing taxon sampling to resolve the phylogeny of baeomycetoid lichens, lichenized taxa with similar secondary chemistry profiles were sampled. The use of secondary chemistry in lichen systematics has a long-standing tradition and in itself presented a key hypothesis to test. Baeomycetoid lichens possess two main classes of secondary compounds, or lichen acids. The Icmadophilaceae contain the depsides baeomycesic, squamatic or thamnolic acid. While the Baeomycetaceae possesses the depsidones stictic or norstictic acid. Two genera of non-baeomycetoid lichens, *Siphula* and *Thamnolia* were noted to possess depsidones and were therefore ideal targets for taxon sampling. The classification of these two genera in the Lecanorales was irresolute and apparently based solely on the character of lichenization. *Siphula* and *Thamnolia* are both nonsexual and not only fail to produce ascospores but also lack any vegetative or mitotic propagules typical of other lichens. The cladistic analyses of nSSU and nLSU rDNA in Chapter three demonstrate that these two genera are closely related and paraphyletic with the baeomycetoid lichens of the Icmadophilaceae. The hypothesis that *Siphula* and *Thamnolia* represent lichenized anamorphs is introduced here and represents a novel concept in fungal systematics.

While working on Chapter three, I discovered that the nSSU rDNA sequence of *Thamnolia subuliformis* I had been using in my data set appeared to be chimeric. This sequence was generated by workers in another laboratory some years previous. Although

the sequence appears to be chimeric, the source of this phenomenon is dubious and may or may not be the result of PCR-mediated chimera formation. Chapter four addresses the behavior and detection of chimeric sequences in phylogenetic analyses. This is the first report of a chimeric or chimera-like fungal nuclear SSU rDNA sequence. The value of this study cannot be over-emphasized as it serves to focus attention on measures of quality control in sequence based phylogenetic studies.

Both Chapters five and six test hypotheses of monophyly of and within the Helotiales. Chapter five examines the evolution of the earth tongue fruitbody morphology and provides convincing evidence that the family Geoglossaceae (the earth tongue family) is not monophyletic. Cladistic analyses of nuclear SSU and LSU rDNA data provide convincing evidence that the earth tongue morphology has been converged upon multiple times throughout the evolution of ascomycetes. This observation parallels the example in hypogeous or truffle-like fungi, in which independent lineages of both ascomycetes and basidiomycetes produce closed fruitbodies which develop (or fruit) below ground and do not forcibly discharge their spores. Furthermore, as shown by other studies (Bruns et al., 1989) the fruitbody form within fungi exhibit great plasticity and are generally not good characters for cladistic studies because of convergent evolution. Chapter five also has important implications for fungal classification as we must reject the hypothesis that the Geoglossaceae are monophyletic. A new classification scheme for these fungi must therefore be proposed, unless we are to accept polyphyly at the family level.

Chapter six includes the broadest taxon and character sampling to infer the phylogeny of inoperculate discomycetes and examine the evolution of life history

strategies within the Helotiales. Because the Helotiales share so many broadly overlapping characters with the Rhytismatales, fungi representing both orders are included in the taxon sampling. In fact, one goal of this study is to better delimit the phylogenetic boundary between the Helotiales and Rhytismatales, two of the major orders of inoperculate discomycetes. Furthermore, this study includes the largest character sampling from three gene regions representing two loci, in order to transcend the typical single gene phylogeny of SSU rDNA. The inability of nuclear SSU rDNA to resolve relationships across the euascomycetes has been discussed previously (Berbee, 1996; Spatafora, 1995) and is indeed demonstrated here again as well. Part of the second largest subunit of RNA Polymerase II (RPBII) is used as a correlative to help resolve and support the phylogenetic relationships inferred in a single gene phylogeny of nuclear SSU rDNA. As has been the case in numerous other studies (Berbee, 1996; Gargas et al., 1995; Spatafora et al., 1998; Stenroos and DePriest, 1998), inadequate taxon sampling of the inoperculate discomycetes leads to topologies exhibiting artifactual monophyly. Thus it is critical that taxon sampling be adequate enough to legitimately test for monophyly of a taxon.

My major objective during the course of this dissertation research has been to use well designed and progressive sampling approaches and thorough, appropriate experimental methods to test and refine phylogenetic hypotheses that will ultimately change the way we view ascomycete evolution. The Helotiales represents the ideal taxonomic assemblage with which to study cladogenic events of the euascomycete radiation because its members are so diverse and exhibit such a broad range of life history strategies. Just a few years ago, the Presidential address for the Mycological

Society of America pointed to "the lack of attention that has been given to this complex order" (Pfister, 1997). I believe this work will provide definitive information regarding the evolution of the inoperculate discomycetes, yet serve as impetus for further examination of the mysteries which remain to be unveiled regarding the evolution of euascomycetes.

1.3 Taxon Sampling

Taxon sampling is a complex and critical issue in molecular phylogenetics (Graybeal, 1998; Kim, 1996; Lecointre et al., 1993; Poe, 1998). Previous nrSSU studies that have included members of the Helotiales have resulted in tree topologies which infer monophyly for the order (Berbee, 1996; Gargas et al., 1995; Spatafora et al., 1998; Stenroos and DePriest, 1998). Such topologies are artifacts of limited taxon sampling. Increasing the density of taxon sampling both within and outside of the Helotiales results in the inference of a polyphyletic order. While, it could be argued that the ultimate taxon sampling of the Helotiales would include nearly all of the some 2,306 species plus a large number of other euascomycetes, such a strategy would not be practical. Furthermore, inferring big phylogenies can also be problematic (Hillis, 1996; Kim, 1998; Yang and Goldman, 1997) and is limited by the phylogenetic methods themselves as well as the number of characters available (Lecointre et al., 1994). A more reasonable approach proposed and used in this study is to choose exemplars from the various groups or subclades (Hibbett et al., 1997). In the SSU rDNA data set (Chapter 6), I have included members of 12 of the 13 families currently recognized in the Helotiales, as well as representatives from most of the other major euascomycete lineages. The density of

taxon sampling was then increased based on inferred topologies and observed branch lengths; including additional taxa which could potentially break up long branches. The rationale for this approach is based on studies suggesting that accuracy of phylogenetic inference is increased when the taxon sampling criterion is based on choosing taxa that will break up long branches (Donoghue et al., 1989; Gauthier et al., 1988; Graybeal, 1998; Hendy and Penny, 1989; Huelsenbeck, 1991; Kim, 1996). Because the Helotiales has been hypothesized to be non-monophyletic (Pfister, 1997), yet all previous molecular phylogenies inferred monophyly, taxon sampling is especially critical.

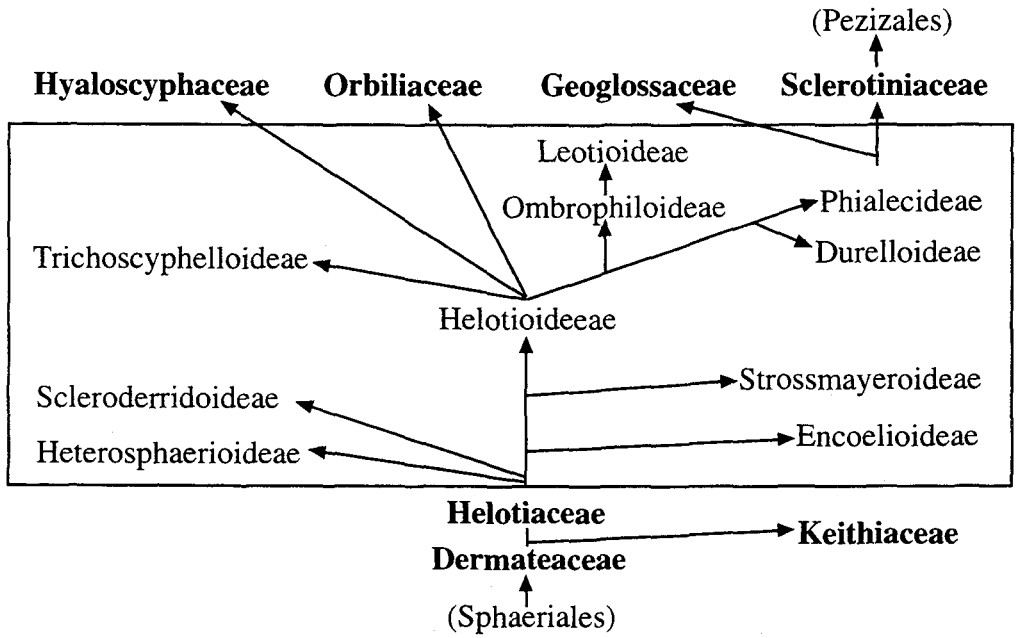
1.3.1 Historical overview of the Helotiales

The order Helotiales is irresolute in many respects. One of the major controversies is nomenclatural. Nannfeldt (Nannfeldt, 1932)(1932) first used the ordinal name Helotiales to encompass six families of inoperculate discomycetes. However, the family Helotiaceae was based on the genus *Helotium* Pers. which is a later homonym of the basidiomycete genus *Helotium* Tode. Thus the type of the genus *Helotium* is a member of the Basidiomycota. Carpenter later proposed the ordinal name Leotiales as a replacement for the Helotiales (Ascomycota) citing articles 10.5, 11.4, and 16.1 of the International Code of Botanical Nomenclature (ICBN) (Carpenter, 1988). However, the botanical code does not require names above the rank of family to be changed in such cases and at the most recent International Botanical Congress (1999), the family name Helotiaceae was conserved (not against Leotiaceae) and so either ordinal name, "Helotiales" or "Leotiales" may be used (unpublished).

The taxonomy of the order is also unsettled. At the familial level Korf (1973) accepted eight families, Eriksson & Hawksworth (1993) accepted 9 families, and Hawksworth et al. accept 13 families with 88 genera being referred to order only (Hawksworth et al., 1995). Many other earlier familial classifications have also been proposed (Dennis, 1968; Gäumann, 1964; Korf, 1958; Nannfeldt, 1932), and others). Figure 1.1 illustrates one of the early hypotheses of interfamilial relationships within the Helotiales (Korf, 1958).

The historically tenuous classification concepts of the order stem from plasticity in the morphological characters which have primarily been comprised of architectural features of the ascus. Furthermore, the morphological characters used to define the Helotiales overlap broadly with characters in other orders of inoperculate discomycetes, particularly the Rhytismatales. Typically, stromata are absent but may be present in the form of sclerotia or a lichenized thallus. The ascomata are apothecial, sessile or stipitate, and composed of simple paraphysoid interascal tissue. The asci are usually small, thin-walled, unitunicate (without separable wall layers), with an apical pore (rather than an operculum). The ascus apex may or may not blue in iodine. Furthermore, some members of the Helotiales lack ascus characters altogether as they are anamorphic. These features have not only contributed to volatile classifications for the Helotiales, but have also made predictions about the phylogenetic relationship to the rest of the euascomycetes difficult.

Figure 1.1 Possible relationships within the Helotiales based on the classification of Nannfeldt (1932), as modified by Korf (1958).



Before Hennig's landmark paper (1965), many phylogenetic hypotheses were put forth regarding the evolution of the Helotiales. These hypotheses were not, of course, based explicitly on synapomorphies. Luttrell's fundamental Helotialean origin hypothesis was that the Helotiales evolved from the pyrenomycetes (or an ancestor of the pyrenomycetes) and subsequently gave rise to the Lecanorales and Rhytismatales (Phacidiales) (Luttrell, 1955) (Figure 1.2). Korf presented a different phylogenetic hypothesis in which the ancestor of the pyrenomycetes ("Sphaeriales") gave rise to the Rhytismatales (Phacidiales) and Helotiales independently (Korf, 1958). But Korf's hypothesis differed markedly from Luttrell's in the Helotiales giving rise to the Pezizlaes (Figure 1.3). These hypotheses are just two examples of the conflict caused by the interpretation of these scanty, broadly overlapping, morphological characters which are also unamenable to the integration of anamorphic or nonsexual species.

Figure 1.2 Luttrell's phylogenetic diagram of the Ascomycota (Luttrell, 1955)).

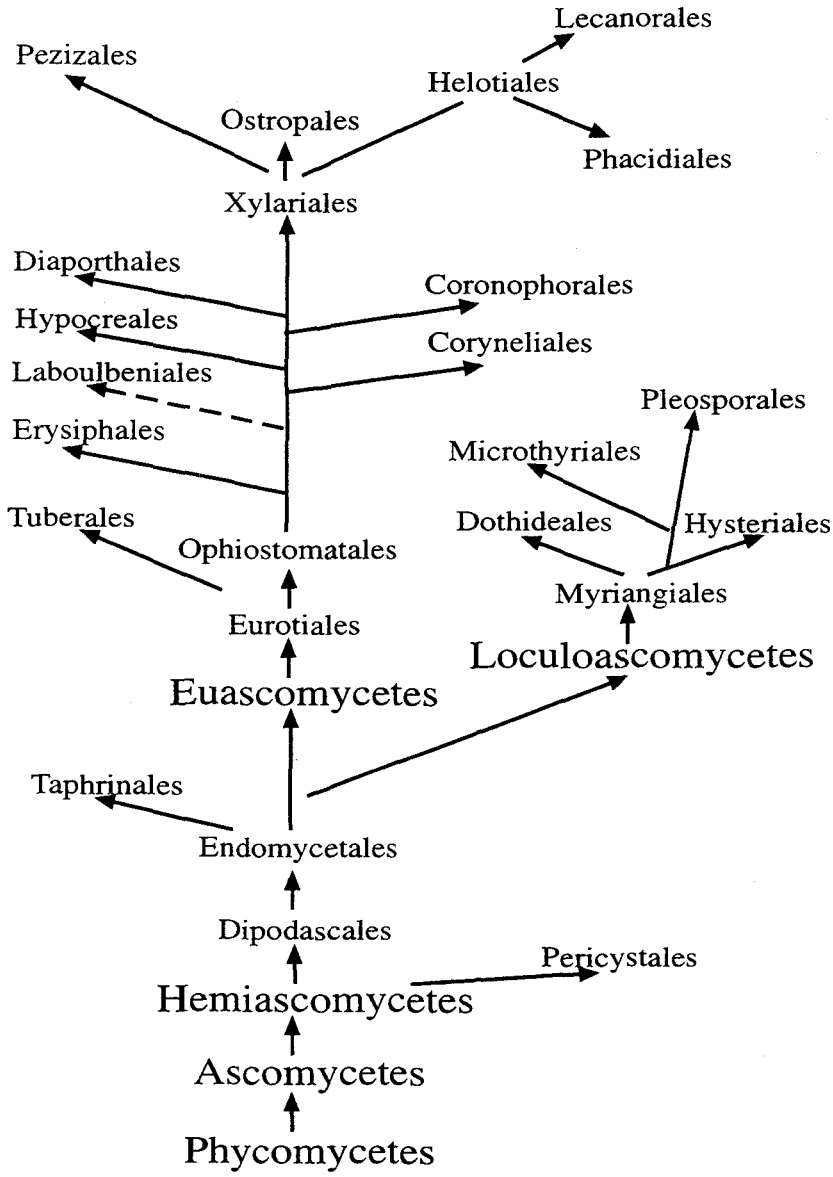
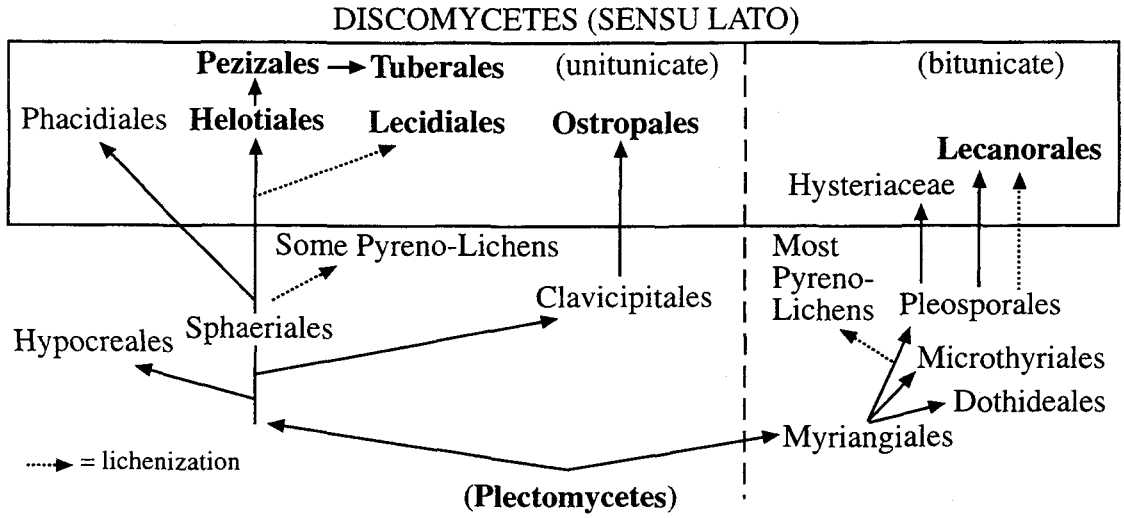


Figure 1.3 Korf's (1958) phylogenetic hypothesis for the Ascomycota.



1.3.2 Evolution of life history strategies of the Helotiales

The Helotiales is a large and complex order of inoperculate discomycetes comprised of at least 2,036 species representing some 392 genera (Hawksworth et al., 1995) . Members of this assemblage have followed divergent evolutionary pathways and display a wide variety of life history strategies. As previously mentioned, some members form symbioses and participate in mycorrhizal or lichen associations. Many members are asymptomatic endophytes of root, leaf, or other plant tissues. Still others are also considered to be important plant pathogens. For example, members of the Sclerotiniaceae cause such serious plant diseases as brown rot of stone fruits, lettuce drop, leaf spot of alfalfa, black spot, and soft rot of onions. These pathogens cause significant agricultural damage and subsequent economic losses each year. Other

pathogens in the Helotiales impact Pacific Northwest forest ecosystems. Canker diseases of western hemlock and Sitka spruce among others, are caused by members of the Helotiales (*Discocainia*). As previously mentioned, members of the Rhytismatales often exhibit similar life history strategies and fill related ecological niches (Table 1.1).

Table 1.1 Summary of the families of Helotiales and Rhytismatales and their general life history strategies (adapted from Hawksworth et al, 1985). * = included in this study.

Helotiales	Life history strategy	Rhytismatales	Life history strategy
?Ascocorticiaceae	saprobic	Ascodichaenaceae	saprobic
Baeomycetaceae*	lichenized	?Cryptomycetaceae	saprobic/parasitic
Dermateaceae*	saprobic/parasitic	Rhytismataceae*	endophytic/saprobic
Geoglossaceae*	saprobic/mycorrhizal		
Hemiphacidiaceae*	endophytic/parasitic		
Hyaloscyphaceae*	saprobic		
Icmadophilaceae*	lichenized		
Leotiaceae*	saprobic/mycorrhizal		
Loramycetaceae*	saprobic		
Orbiliaceae*	saprobic/lichenized?		
Phacidiaceae*	saprobic/parasitic		
Sclerotiniaceae*	pathogenic/saprobic		
Vibrisseaceae*	saprobic		

The reproductive mode of Helotialean fungi is also diverse. While many members reproduce sexually by producing meiotic ascospores within asci, some members are strictly asexual and reproduce via mitotic spores. The anamorphs (mitotic fungi) known from the Helotiales are varied but are generally hyphomycetous or coelomycetous (Hawksworth et al., 1995). Coelomycetous anamorphs produce conidia (mitotic spores) within a cavity lined with fungal or fungal and host tissue. The hyphomycetous anamorphs generally have more complex conidiogenous events which result in generally more elaborate conidiomata. Almost 40 years ago, Kimbrough wrote,

"Surprisingly, very little work has been done on the sexuality and development of members of the Helotiales, considering the very large number of species involved." (Kimbrough, 1970). Using the ubiquitious characters of molecular sequence data, sexual and asexual lineages can be compared and used to form evolutionary hypotheses integrating these fungi with such varied reproductive strategies.

Because the polyphyletic assemblage of fungi classified in the Helotiales do exhibit such diverse life history strategies, examining the evolution of these lineages and determining various cladogenic events will ultimately reveal much about ascomycete evolution in general. Furthermore, cladistic analyses of sequence data may reveal "good" morphological, ecological, and ontogenetic characters within certain Helotialean groups which can then be used with molecular data to produce more robust, better resolved organismal phylogenies at the family level or below.

1.4 Character Sampling

Character sampling is a critical consideration in any phylogenetic study. The number and quality of characters is crucial to inferring robust, well-resolved phylogenies (Jorgensen and Cluster, 1989; Lecointre et al., 1994). Characters must be variable enough to provide phylogenetic signal, yet conserved enough to allow one to assess homology across the taxa sampled. As discussed above, the plentitude of convergent evolution within the Fungi has been an impediment to traditional systematic studies which have most frequently been based on morphological characters. Furthermore, morphological or ecological characters alone are not adequate for forming evolutionary hypotheses or natural classifications within the Helotiales since they restrict integration

of lichenized and nonlichenized, sexual and asexual taxa. At best, the amount of cross-comparative morphology available is miniscule and the total number of morphological characters is inadequate for any phylogenetic study incorporating an acceptable taxon sampling. However, the confines presented by the diverse morphologies of Helotialean fungi are easily overcome with the use of molecular data. The objective of this study is to reconstruct the phylogeny of members of the Helotiales and examine evolutionary pathways based on sequence data from four gene regions representing three independent loci. The loci utilized in this study are introduced below, along with the rationale for sampling these characters.

1.4.1 Nuclear ribosomal DNA

Nuclear ribosomal DNA is unquestionably the gene region used most frequently in molecular fungal systematics. Nuclear rDNA is non-translated and ultimately functions as RNA scaffolding for ribosomes. It is not a gene *per se*, but rather a region of tandem repeat units. There are several reasons for the widespread use of nuclear rDNA. 1) It is ubiquitous. Because it is found in all organisms it allows for broad comparisons, even inter-kingdom comparisons if a highly conserved region of nuclear rDNA is used (e.g., 5.8S). 2) Consequently, there are many nuclear rDNA sequences available from databanks such as GenBank and EMBL. Results from different labs can be compared and available sequences can be used by others, eliminating the need for access to specimens, cultures, reamplification and resequencing of taxa. This also facilitates testing reproducibility, a fundamental aspect of basic science. 3) It is present in many copies per cell. The typical fungal cell is estimated to have between 100-200 rDNA

repeat units. Yet, despite this high copy number the locus basically behaves like a single copy gene (Bruns et al., 1991) (Bruns et al., 1991). 4) The rDNA repeat unit has regions which evolve at different rates. Therefore, different portions of the gene region can be used for developing phylogenetic hypotheses at various taxonomic levels. 5) This region also provides a fairly large number of characters for phylogenetic analyses. 6) Furthermore, as with other gene regions, the mode of sequence variation can be observed (transition vs. transversion, etc.) and the degree of nucleotide bias can be measured and incorporated into phylogenetic analyses or evolutionary models (Bruns et al., 1991).

1.4.1.1 *Nuclear SSU ribosomal DNA*

Nuclear Small-Subunit rDNA (SSU nrDNA) is the most conserved region of the rDNA repeat and has most frequently been the region of choice in molecular fungal systematics. Despite the disproportionate amount of fungal systematic literature based on SSU rDNA sequence data, it is important to note that this region, like others, has limited resolution. Specifically, SSU rDNA is most efficient at resolving relationships above the genus-level. However, it fails to provide resolution along the euascomycete backbone (Berbee, 1996; Spatafora, 1995) especially when taxon sampling is large and heterogeneous.

Kumar and Rzhetsky (Kumar and Rzhetsky, 1996) recently used SSU rDNA to examine relationships of the eukaryotic kingdoms. Although theirs was not the first study examining inter-kingdom relationships involving Fungi (Cavalier-Smith, 1993; Gouy and Li., 1989; Smothers et al., 1994; Van de Peer et al., 1993; Wainwright et al., 1993) they were able to produce what they termed "a reliable branching order of

eukaryote phyla" in the "crown" of the tree using distance methods. They based this "reliable branching order" on the results of four-cluster analysis (which evaluates statistical confidence of branching order) yielding confidence probabilities of 90% or greater. Furthermore, they took the molecular and evolutionary properties of SSU nrDNA into account when computing statistical confidence. Four-cluster analysis allowed them to account for biological information like 1) the nonindependence of evolutionary changes in the stem regions of the SSU rRNA gene and 2) heterogeneity of evolutionary rates among sites. Their study also contained a significant sampling, including 45 species of fungi from 35 genera. They confirmed that 1) animals and true fungi are sister groups and 2) the red algae are the closest relatives of animals, true fungi, and plants, with heterokonts basal to these. They did conclude however, the SSU nrDNA sequence data was not sufficient to resolve the branching order of eukaryotic lineages that diverged prior to the evolution of alveolates, which appeared basal to the heterokonts.

Wainright and co-workers (1993) also used SSU rDNA to examine inter-kingdom relationships but used maximum-likelihood methods for analyses. The number of taxa they sampled was significantly less and only included four fungal species and no red algae. However, they were also able to infer that animals and fungi share a unique evolutionary history. It is difficult to compare the amount of resolution they obtained with that obtained by Kumar and Rzhetsky (1996) because the sampling schemes differed so significantly. From these studies we can see that although SSU nrDNA sequence data may be used for inter-kingdom comparisons, it still may not provide sufficient resolution

for certain ancient-diverging lineages or rapid radiations, such as that of the euascomycetes.

Phyla level comparison using SSU nrDNA are quite common in the fungal systematics literature and SSU nrDNA appears to be better at resolving phyla-level relationships than inter-kingdom ones. Bowman and co-workers (1992) used SSU nrDNA nucleotide sequence data to look at evolutionary relationships between the Ascomycetes, Basidiomycetes and Chytridiomycetes. They used two primary methods of phylogenetic analysis, Neighbor-Joining (Saitou and Nei, 1987) and parsimony-based Winning Sites Test (Prager and Wilson, 1988), which determines how strongly the branching order is supported by the data. They concluded that SSU nrDNA data which contains a significant number of characters, is able to resolve relationships at this level better than 5S rDNA which had been used previously (Hori and Osawa, 1987; Van de Peer et al., 1993) (Van de Peer, et al, 1993; Hori and Osawa, 1987). The Bowman et al. results seem to be resolved with a relatively high degree of confidence and also support the morphological classification uniting the two "classes" of nonflagellated fungi.

Later, Bruns et al. (1992) combined 14 SSU nrDNA sequences from independent work done in four laboratories to examine phylum-level relationships with the Fungi. Their inferred phylogenies, using both distance and parsimony methods, supported 1) the basal position of Chytridiomycetes and Zygomycetes, and 2) the derived, monophyletic nature of Ascomycetes and Basidiomycetes. In addition, they resolved some lower-level relationships including 1) support of Ascomycete traditional order classification and division of the hemi- and euascomycetes into distinct lineages, and 2) Basidiomycete division between holobasidiomycetes and phragmobasidiomycetes. The phylum-level

relationships were well-supported and were robust to minor changes in alignment, weighting, and method of analysis.

A more recent paper using SSU nrDNA to deal with phylum-level fungal systematics is that of Berbee and Taylor (1993). This paper is particularly interesting because they attempt to construct a relative time scale for the origin and radiation of Fungi. They reconstructed the phylogeny of Fungi using SSU nrDNA sequence data and then calibrated a relative time scale using fossil evidence. Because the different fungal lineages can have different nucleotide substitution rates (Bruns et al., 1992), they normalized the pairwise substitution data before estimating the relative time of divergence. They found evidence for a substitution rate of 1% per lineage per 100 MY and were able to hypothesize divergence of terrestrial fungi from chytrids approximately 550 MYA. They further were able to estimate the ascomycete-basidiomycete split as occurring around 400 MYA.

SSU nrDNA nucleotide sequence data has perhaps been most frequently used for inter- and intra-ordinal level relationships (Eriksson and Hawksworth, 1996; Gehrig et al., 1996; Hausner et al., 1992; LoBuglio et al., 1996; Nagahama et al., 1995; Saenz, 1994; Spatafora, 1995; Spatafora and Blackwell, 1993; Spatafora and Blackwell, 1994). The amount of literature from fungal systematic research using SSU rDNA at these levels is disproportionately great. Perhaps it is because at these levels we can ask some of the most interesting evolutionary questions with regard to symbioses, ontogeny, nutritional modes, and reproductive modes. For example, Gargas and co-workers (1995) (Gargas et al., 1995) looked at the evolution of fungal symbioses using SSU rDNA sequence data. Their parsimony based analysis suggested at least five independent origins of the lichen

symbiosis – three origins within the basidiomycetes and two within the ascomycetes. By using the assumption that evolution proceeds from a saprobic to symbiotic nutritional mode then from parasitism to mutualism, they tried to examine the evolutionary progression of symbiotic relationships. They were not able to find support for the idea that symbiotic organisms evolve from parasitism toward mutualism. This lack of support however, was probably not due to the resolving power of SSU rDNA, but due more to the limited sampling and nature of the question being asked.

Another interesting aspect of using SSU nrDNA, especially at the ordinal-level is that rRNAs (coded for by rDNA) often possess "signatures" (Eriksson, 1995) (Eriksson, 1995). Eriksson analyzed stem-loop E23-1 in several ascomycetes and found putative signatures in the helix of the terminal portion. This, as well as other secondary structural aspects, may facilitate confirming homology for various characters. In the following chapters, predicted secondary structure was used to facilitate assessment of positional homology in sequence alignments.

Because of the availability of nuclear SSU rDNA sequences on GenBank, this molecule served as the cornerstone for the phylogenetic studies of this dissertation research. Furthermore, this molecule has been the single most widely utilized gene region in fungal molecular systematics and has been demonstrated to be effective at resolving some inter- and intra-ordinal relationships. In addition, the availability of fungal-specific primers facilitated amplification of fungal SSU rDNA sequences without concomitant amplification of symbiont DNA (algal, cyanobacterial, or plant) and thus the time-consuming process of cloning was negated. Finally, the value of generating additional nuclear SSU rDNA sequences for inclusion in other phylogenetic studies,

cannot be overstated. With the availability of more Helotialean nuclear SSU rDNA sequences on GenBank, more accurate phylogenies may be inferred and the artificial monophyly of the order observed in previous studies will be a thing of the past.

1.4.1.2 *Nuclear LSU ribosomal DNA*

The large subunit (LSU) portion of the ribosomal RNA gene is a mosaic structure (like other rRNA genes) being composed of conserved core segments and hypervariable expansion segments. The core segments are defined by homologies in prokaryotic rRNA genes and are likely essential to ribosome functions (Noller, 1984; Gerbi, 1985) . The core regions therefore evolve more slowly due to the evolutionary constraints of function. In contrast, the expansion segments, or divergent domains have no precise equivalents in prokaryotes and exhibit greater sequence divergence (Gerbi 1985; Clark, 1987). Because of this mosaic structure, nuclear LSU rDNA has been used at a variety of levels.

The large subunit (LSU) of the nuclear rDNA, or 28S rDNA, has been used much like SSU rDNA and for similar phylogenetic studies at similar taxonomic levels. However, the amount of literature from studies of LSU-based fungal phylogenies is significantly less than that of SSU-based phylogenetic studies. Despite large subunit (23S and 23S-like) rDNA databanks and compilations of rRNA secondary structures, which continue to grow, there are still fewer fungal LSU rDNA sequences published in relation to SSU rDNA sequences (Gutell et al., 1992) and this is especially true for lichenized taxa. However, as the utility of LSU sequence data becomes more apparent in fungal systematics, more fungal LSU sequences should become available. LSU rDNA may be particularly useful because secondary structure can aid in determining

homologous characters and thus facilitate alignment in variable regions of the molecule, like SSU rDNA, but it may provide more potentially parsimony-informative characters for relatively less sequencing effort than SSU rDNA.

Qu and co-workers (1988) used 400 nucleotides from the 5' end of the LSU to examine eukaryotic phylogeny. Although this study did not significantly and directly impact fungal systematics, since they only included one fungal species, it was one of the first to examine these higher order relationships involving Fungi using LSU rDNA sequence data. They emphasized in this work that even a partial sequence of LSU rDNA of an appropriate region may suffice to generate reliable phylogenetic estimations and that LSU rDNA has the potential to facilitate generation of phylogenetic hypotheses for both higher and lower level relationships. They employed both distance and parsimony techniques in concluding that this conserved domain of the LSU has a relatively uniform rate of nucleotide substitution and is sufficient for addressing questions at the level of the eukaryotic lineages examined.

More recently, nuclear LSU rDNA is being used in ascomycete phylogenetic studies although it has not seen the popularity of SSU rDNA. Generally the first 600-900 bp are used most often. This region usually includes the divergent domains D1 through D3. As is often the case, support for phylogenetic hypotheses can often be enhanced by using a combination of gene regions for nucleotide sequence data. This is especially true with rDNA where the SSU and LSU data are being used more frequently in the same study to enhance resolution and increase robustness of phylogenetic hypotheses. Several recent papers use combined SSU and LSU rDNA to examine phylogenetic relationships within ascomycete families and orders (Holst-Jensen et al., 1997; Spatafora et al., 1998,

and others). These works generally support the hypothesis that increasing the number of characters can help infer more resolved phylogenies.

1.4.2 RNA Polymerase II (RPB2)

RNA Polymerase II (*RPB2*) is a single copy nuclear gene which encodes the second largest subunit of nuclear DNA-dependent RNA polymerase II. This polymerase is the key enzyme that transcribes pre-mRNA. The exploitation of this suite of characters is novel to fungal systematics. The use of RPB2 was recently initiated as a suitable alternative to the commonly used SSU rDNA region in fungal molecular systematics (Liu et al., in press (1999)) . This molecule has several qualities which make it a good candidate for use in molecular phylogenetic studies; namely, it is a relatively slowly evolving single copy nuclear gene of considerable size (over 2.5 kb). Furthermore, since it is a protein-coding gene, inferred amino acid sequences can be used and are predicted to be less susceptible to problems of long branch attraction and codon bias than nucleotide sequences of rDNA (Felsentstein 1996). Both of the largest subunits of RNA polymerase II, *RPB1* and *RPB2*, have been used in broad-scale evolutionary studies and have proven to be useful for inferring phylogenies of eukaryotes (Iwabe et al., 1991; Sidow and Thomas, 1993; Klenk et al., 1995; Stiller and Hall 1997; Denton et al., 1998). Furthermore, it is becoming evident that any robust and comprehensive phylogenetic study must use information from more than a single locus to infer a phylogeny. Because PCR and sequencing technology has improved greatly over the past several years, it takes relatively less effort to generate more sequences than it did with manual sequencing. It is also critical that we begin to move away from single gene phylogenies, based solely on

rDNA, and move toward more complete organismal phylogenies by using multiple sources of data – whether it be molecular data from other loci, or morphological, ecological, and ontological data as well.

While other protein-coding genes, such as Beta-Tubulin or Elongation Factor 1-alpha, have seen some recent use in ascomycete systematics these have typically been studies at lower taxonomic levels. One challenge regarding this dissertation work is to find a protein-coding gene which can be used across the ascomycetes. Amino acid sequences of β-tubulin have been used for comparison of phylogentic relationships across kingdoms however, obtaining nucleotide sequences via PCR and direct sequencing can be somewhat labor-intensive. One difficulty which arises is that β-tubulin is not a single copy gene. This trait became an issue in this dissertation study when PCR amplification with the most commonly used β-tubulin primers yielded multiple bands in both lichenized and nonlichenized taxa. Furthermore, recent work suggests that β-tubulin has undergone a gene duplication event within the Archiascomycetes (Landvik, unpublished), which can greatly confound problems of phylogenetic analysis because of comparisons of paralogous rather than orthologous genes (Hillis et al., 1996). This problem also arises when genes are part of gene families, as is the case with Chitin Synthase (Chua et al., 1994) .

1.5 Phylogenetic Analyses

Evolutionary reconstructions based on the molecular characters discussed above are carried out principally based on optimality criteria. Specifically, maximum parsimony or weighted parsimony criteria are employed most frequently, however, in

some cases algorithmic methods are used to test or support hypotheses. Maximum Likelihood methods are also used in some cases, especially when choosing which of several or many equally most parsimonious trees to discuss. Kishino-Hasegawa Maximum Likelihood ratio tests are used as a statistical, parametric method for testing null hypotheses. Each of the following chapters include detailed information regarding the phylogenetic methods used to infer phylogenies. It is my intent to use the most robust methods available to infer nucleotide based phylogenies which will greatly contribute to our understanding of the relationships surrounding and within the inoperculate discomycetes. However, methods of phylogenetic analyses are highly debated and each is likely to have it's own set of weaknesses.

1.6 Conclusions

Perhaps a significant reason for the surge in fungal molecular systematic research, apart from the attractiveness of the number of characters generated, is that in general, fungal lifestyles lend themselves very nicely to forming evolutionary hypotheses. Fungi seem to repeat several major patterns of convergence regarding symbioses, nutritional and reproductive modes, and morphology. This plasticity of phenotype makes assessment of homologous morphological or ecological characters difficult. This is certainly the case regarding the diverse and complex group of inoperculate discomycetes comprising the Helotiales and Rhytismatales. With molecular data phenotypic plasticity is no obstacle and it is often possible to address several evolutionary questions at the same time. For example, the vast array of ascomycete symbioses is particularly interesting and may allow for the generation of evolutionary hypotheses not only about

fungi but also about their symbionts. The variety of life history strategies and reproductive modes within the inoperculate discomycetes also makes it possible to polarize characters that appear to be major evolutionary steps, such as host-shifts, changes in nutritional mode, lichenization, and loss of sexual reproduction.

Despite these provocative aspects of inoperculate discomycete biology they have largely been ignored in molecular phylogenetic studies. In addition, relatively few genes have been used as sources of phylogenetic sequence data for ascomycete systematics. Perhaps because relatively few details are known about most fungal genomes. This study is unique and significant because it not only includes such a dense sampling of inoperculate discomycetes, but it also utilizes multiple, independent loci to make phylogenetic inferences regarding the evolution of this unique and beautiful group of organisms.

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CHAPTER 2

A Re-examination of Generic Concepts of Baeomycetoid Lichens Based on Phylogenetic Analyses of Nuclear SSU and LSU Ribosomal DNA

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2.1 Abstract

The lichen symbiosis has evolved multiple times within the Kingdom Fungi, although the total number of lichenization events leading to extant taxa is still unclear. Two lichenized families, the Icmadophilaceae and Baeomycetaceae have been classified in the Helotiales. Because the Helotiales are predominantly non-lichenized, this suggests that these families represent independent evolutionary episodes of lichenization from the Lecanorales. As a first step toward understanding the evolution of the lichen symbiosis within this order, we tested recent hypotheses concerning the segregation of lichen genera between the two lichen families. Specifically, we used phylogenetic analyses of nucleotide sequence data from nuclear small-subunit and large-subunit ribosomal DNA to test the morphology-based hypotheses that *Dibaeis* is a distinct genus from *Baeomyces* and that *Dibaeis* is a member of the Icmadophilaceae rather than the Baeomycetaceae. Phylogenetic analyses of nuclear SSU rDNA and combined SSU and LSU rDNA data support the hypothesis that *Dibaeis* is more closely related to *Icmadophila* than it is to *Baeomyces*. Therefore, these data support the resurrection of *Dibaeis* from its previous synonymy with *Baeomyces* based on the characters of ascocarp color and ascus morphology. The recognition of two distinct genera is also consistent with character state distribution of unique lichen acids.

2.2 Introduction

It has been proposed that the Baeomycetaceae and Icmadophilaceae represent parallel evolutionary lines within the order Helotiales (Rambold et al., 1993). In fact, these are the only lichenized families currently placed within this large and heterogeneous order of inoperculate discomycetes and as such represent an independent and potentially more recent event of lichenization than the Lecanorales. The systematic position of the core genera in both the Baeomycetaceae and Icmadophilaceae has been debated for over a

century and has recently been re-examined using morphological data (Gierl and Kalb, 1993; Rambold et al., 1993).

The Baeomycetaceae contains the genera *Baeomyces* and *Phyllobaeis* and the Icmadophilaceae contains five genera; *Dibaeis* , *Icmadophila* , *Knightiella* , *Pseudobaeomyces* , and *Siphulella* (Hawksworth et al., 1995). The names *Dibaeis* and *Baeomyces* were treated as synonyms until 1993 when Gierl & Kalb resurrected *Dibaeis* to include those species of *Baeomyces* with rose colored ascocarps. *Baeomyces* is retained for those species with brown ascocarps.

Both lichen families have characters that overlap in many respects. These characters include a typically crustose to squamulose thallus with sessile or shortly stipitate flat or convex apothecia which may be clustered on the thallus. Apothecia are formed on specialized often non-lichenized thalline branches. The paraphyses are generally simple or sparsely branched and are often swollen at the apices. The ascospores are hyaline and simple or transversely septate. Both families have pycnidial anamorphs and have green algal photobionts. While both families possess asci with the "eversion-type" of dehiscence warranting their consideration in the Helotiales (Chadefaud, 1960; Duvigneaud, 1944; Honegger, 1983; LeGal, 1946; Rizzini, 1952),the major delimiting characters for the separation of these two families is the shape of the ascus apex and the iodine reaction (+/-) of the apical cap (Rambold et al., 1993).

Until recently, lichen systematics has relied heavily and almost exclusively on morphological and chemical characters. While these types of characters can be quite useful for elucidating phylogenetic relationships, they are often difficult to compare across a broad range of taxa and are not often amenable to comparisons of lichenized and non-lichenized fungi. Gierl & Kalb's reintroduction of *Dibaeis* and the subsequent reclassification of this genus from the Baeomycetaceae to the Icmadophilaceae (Rambold et al., 1993) were largely based on comparisons of ascus ultrastructure. A major objective of this research was to test these hypotheses based on morphological features with phylogenetic analysis of

nucleotide sequence data from the nuclear small-subunit (SSU) and large-subunit (LSU) ribosomal DNA (rDNA).

2.3 Materials and Methods

2.3.1 Sequence Determination

For the nuclear SSU and LSU rDNA sequences determined in this study, total genomic DNA was extracted from eight freshly collected specimens by a modified CTAB method (Gardes and Bruns, 1993) after two to three acetone extractions to remove lichen secondary compounds. Voucher specimens are deposited in the OSC Mycological Collection at Oregon State University (Table 1). Unless otherwise indicated, all reagents were obtained from Sigma Chemical Company, St. Louis, MO. Samples were ground with liquid nitrogen immediately before the addition of 2X CTAB buffer pre-warmed to 65°C and supplemented with 2% β-mercaptoethanol. One phenol:chloroform (1:1) followed by two chloroform:isoamyl alcohol (24:1) extractions preceded Isopropanol precipitation of DNA. Following crude extraction, DNA was further purified using the Elu-Quik DNA Purification Kit (Schleicher & Schuell, Inc., Keene, NH)

Polymerase chain reactions (PCR) were performed in 50 µl reaction volumes that contained approximately 50 ng of DNA, 10mM Tris-HCl, pH 8.3, 50mM KCl, 0.005% Tween 20, 0.005% NP-40, 1.5mM $MgCl_2$, 62.5 µM dNTP mix (containing equal amounts of each of the four deoxyribonucleotide triphosphates), 0.5 µM of each PCR primer, and 2.5 units Replitherm™ Thermostable DNA Polymerase (Epicentre® Technologies, Madison, WI). Nuclear SSU rDNA was amplified with primers NS17UCB - NS22UCB and NS17UCB - NS24UCB (Gargas and Taylor, 1992) (nu-SSU-0072-5' and nu-SSU-1293-3' and nu-SSU-1750-3' respectively, following the nomenclature recently proposed

by Gargas and DePriest (1996). PCR reactions were overlaid with mineral oil and placed in an MJ Research, Inc. PTC-100™ Programmable Thermal Controller and subjected to the following conditions: 94°C for 3 minutes, 35 cycles of 94°C for 1 minute, 51°C for 30 seconds, and 72°C for 2 minutes then 5 cycles of 94°C for 1 minute, 53°C for 30 seconds, then 72°C for 2 minutes plus 5 seconds per cycle, and finally 72°C for 5 minutes.

LSU nrDNA PCR reactions utilized the primer pairs LROR and LR5 (Vilgalys and Sun, 1994). All reaction concentrations were identical to those used in the SSU rDNA reactions with the exception of final primer concentration which was 0.3 μM. The cycling conditions of PCR were: 94°C for 3 minutes, 39 cycles of 94°C for 1 minute, 48°C for 30 seconds, and 72°C for 45 seconds, and 3 minutes at 72°C.

PCR products were visualized on a 1% agarose gel stained with ethidium bromide and quantified using Gibco-BRL low DNA Mass™ Ladder. PCR products were purified by adding one half volume of 4.5 M ammonium acetate to the PCR product, followed by brief vortexing, then addition of two volumes isopropyl alcohol. This mixture was vortexed briefly and then allowed to precipitate at room temperature for 15 minutes. Each sample was then centrifuged at 10,000g and rinsed with 70% ethanol. The purified pellet was resuspended in the appropriate volume of doubled distilled water to yield a final template concentration of 20-25 ng/μl. The purified product was sequenced on an ABI Prism™ Model 377 (version 2.1.1) automated DNA sequencer (Perkin-Elmer) at the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University. Both the coding and template strands of the double-stranded PCR products were sequenced utilizing the sequencing primers NS2, SR7R, SR11R, NS4,

NS5, and NS24 for SSU nrDNA (White et al 1990; Spatafora et al 1995; R. Vilgalys, unpublished) and LR0R, LR3 for LSU nrDNA (Valgalys and Hester, 1990).

2.3.2 Taxon Sampling

Sixteen species are included in this study. Two specimens of *Baeomyces rufus* are included, one of which was found on the typical substrate, soil, the other of which was found on the atypical substrate, wood. Eight nuclear SSU rDNA and seven nuclear LSU rDNA sequences were obtained from GenBank and are listed alphabetically in Table 2.1 along with those sequences determined in this study. The nuclear SSU rDNA sequence of *Siphula ceratites* was obtained from GenBank while the nuclear LSU rDNA sequence was determined during this study from herbarium material. Thus, it should be noted that these two sequences were obtained from different specimens.

Siphula ceratites was included as an ingroup taxon based on previous cladistic studies which placed it in a monophyletic clade with *Icmadophila ericetorum* and *Dibaeis baeomyces* (Platt and Spatafora, 1997; Stenroos and DePriest, 1998). In order to directly compare inferred phylogenies from SSU rDNA and combined SSU and LSU rDNA sequences, taxon sampling was duplicated in both data sets. Thus, any potential incongruencies could be interpreted as effects of character sampling, rather than artifacts of differential taxon sampling. Therefore, the only members of the Lecanorales included are *Evernia prunastri* and *Loxosporopsis corallifera*, for which we have determined both SSU and LSU rDNA sequences.

2.3.3 Sequence Alignment and Phylogenetic Analysis

Sequences were aligned by visual, color-based estimation using SeqApp (version 1.9a169, Gilbert, 1992). All sequences were edited manually, comparing the computerized sequence

Table 2.1. Taxa included in this study.*

Taxon	OSC #	GenBank # nSSU rDNA	GenBank # nLSU rDNA
Baeomyces rufus‡	56396	AF107347	AF107558
Baeomyces rufus	56397	AF107348	AF107559
Baeomyces placophyllus	56398	AF107349	AF107560
Cudonia circinans	56399	AF107343	AF107553
Dibaeis baeomyces	56400	AF107345	AF107555
Discina macrospora	NA	U42651	U42678
Evernia prunastri	56404	AF107351	AF107562
Gyromitra esculenta	NA	U42648	U42675
Hypocrea lutea	NA	U32407	U00739
Icmadophila ericetorum	56401	AF107346	AF107556
Loxosporopsis corallifera	56405	AF107350	AF107561
Neurospora crassa	NA	X04971	U40124
Ophiostoma piliferum	NA	U20377	U47837
Sclerotinia sclerotiorum	NA	L37541	Z73762
Siphula ceratites	56402§	U72712	AF107557
Spathularia velutipes	56403	AF107344	AF107554
Tuber gibbosum	NA	U42663	U42690

*The Oregon State University Mycological Collection accession number (OSC #) is given for the source specimens for sequences determined in this study. GenBank Accession numbers are given for all SSU and LSU sequences utilized.
‡Specimen of *Baeomyces rufus*_ collected from atypical substrate
§Specimen corresponds to nrLSU sequence only

read to that shown on the color electropherogram. In this way, misreads were occasionally found and corrected. Introns were excluded from alignments and analyses. Ambiguous

nucleotides were assigned an IUPAC standard nucleic acid code (n, r, k, etc.). Gaps in the SSU rDNA data set and the SSU rDNA portion of the combined data set were treated as a fifth character state in order to better utilize potential phylogenetic information present in these indels (Bruns et al., 1992). Those SSU rDNA positions with known nucleotides but which were ambiguously alignable with respect to bordering positions were treated as missing data. This method is based on the recoding procedure of Bruns et al. (1992). Regions longer than four nucleotides with ambiguous alignment were excluded. Thus the divergent domains of the LSU rDNA data set were omitted from phylogenetic analyses. The SSU rDNA data set comprised 1137 total characters. The combined SSU and LSU rDNA data set comprised 1656 total characters.

Phylogenetic analyses were performed using PAUP*4.0d64 (Swofford, 1997). Maximum parsimony, weighted parsimony and maximum likelihood criteria were utilized. Unweighted parsimony analyses were performed on the SSU rDNA and combined data sets using the branch-and-bound search option in PAUP*. Bootstrap values were generated using 1000 replicates of a "fast" stepwise sequence addition with the mulpars option in effect. In both data sets, transition:transversion ratios were estimated using MacClade version 3.0 (Maddison and Maddison, 1992). For weighted parsimony analyses of the SSU rDNA and combined data sets, transversions were weighted 1.5:1 over transitions. Weighted parsimony was used on the combined data set because it may present a more accurate model by down-weighting the more variable, and presumably more homoplastic transitions and thus is useful in increasing the resolving power of parsimony analyses or reducing the total number of equally most parsimonious trees (Swofford et al., 1996).

Maximum likelihood analyses were performed under the Hasegawa-Kishino-Yano (1985) model with a transition:transversion ratio of 1.5:1 and other default options in PAUP* with no enforcement of a molecular clock. Outgroup selection of the Pezizales was based on previous studies which place this clade near the base of the Euascomycetes

(Gargas et al 1995; Spatafora 1995). Choice of Pezizales or pyrenomycete outgroup was equivocal with respect to tree length and ingroup topology.

A partition homogeneity test (PHT) was implemented in PAUP* using 1000 replicates and used to evaluate combinability . As additional measures of statistical support for the segregation of *Dibaeis* from *Baeomyces*, Kishino-Hasegawa and Templeton tests implemented in PAUP* were performed on the combined data set with the topological constraints of the null hypothesis (H_0) enforced (Swofford, 1997). Kishino-Hasegawa tests (Kishino and Hasegawa, 1989) were performed under the Hasegawa-Kishino-Yano (1985) maximum likelihood model.

2.4 Results and Discussion

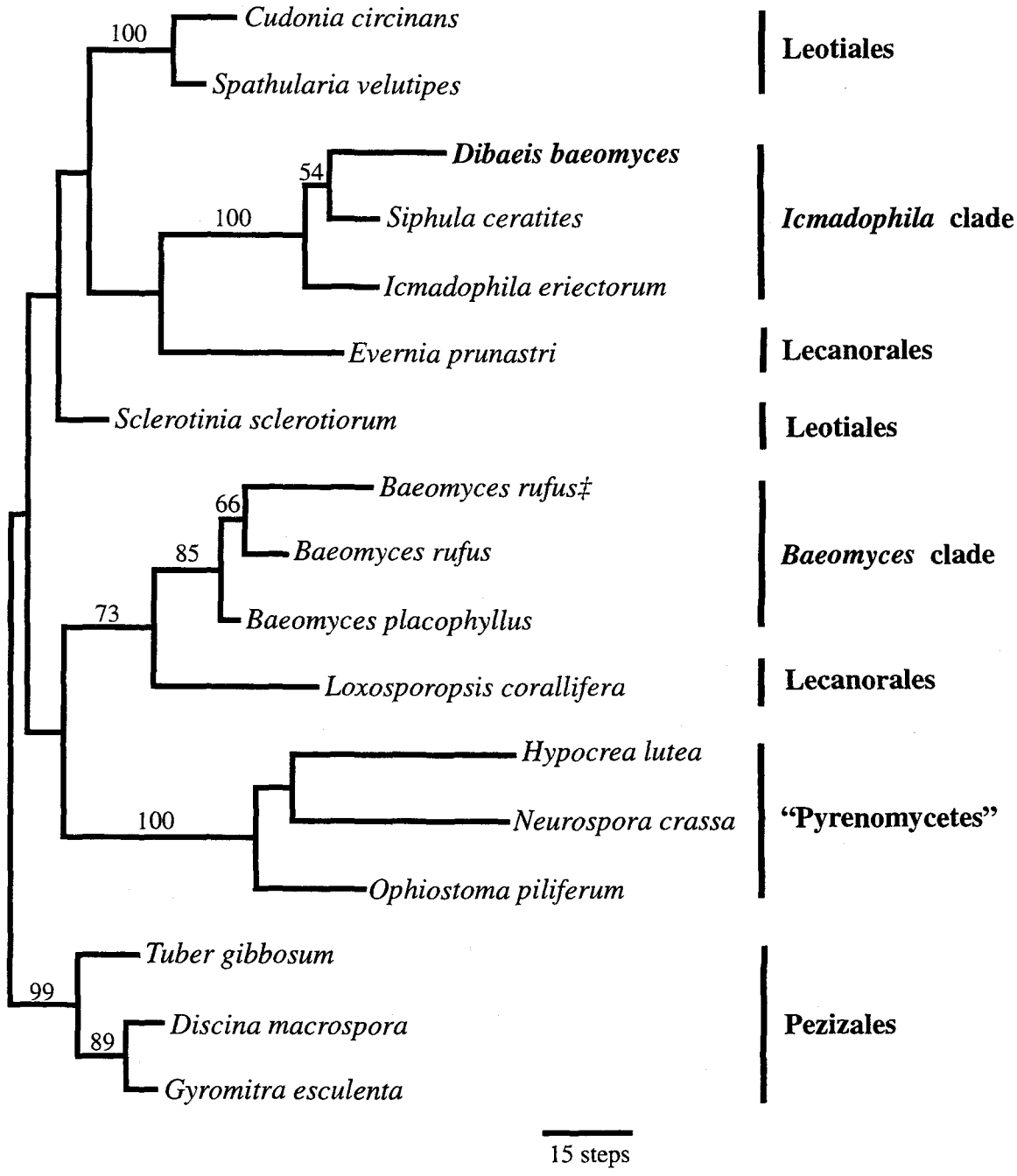
Maximum parsimony analysis of SSU rDNA produced a single most parsimonious tree of 422 steps with a consistency index (CI) of 0.754 and a retention index (RI) of 0.710. Of the 1047 included characters in the nuclear SSU rDNA data set, 134 were potentially parsimony-informative. Weighted parsimony and maximum likelihood analyses of the SSU rDNA data set yielded the identical topology as the tree shown in Figure 2.1 (data not shown). *Icmadophila ericetorum*, *Dibaeis baeomyces* and *Siphula ceratites* formed a monophyletic clade – *Icmadophila* clade – with bootstrap support of 100 (Fig 2.1). The species of *Baeomyces* sampled were not members of this group, rather they formed a distinct well-supported clade. Under this taxon sampling scheme, *Baeomyces* and the *Icmadophila* clade are not sister groups, i.e., the two taxa do not form a monophyletic clade. The nonlichenized Helotiales comprise a paraphyletic lineage, with *Sclerotinia sclerotiorum* occupying the basal position within the group. The *Icmadophila* clade grouped with *Evernia prunastri* within the paraphyletic assemblage of Helotiales. However, there is no bootstrap support for the sister group relationship of *Evernia* with the Icmadophila clade and this topology is most likely an artifact of the paucity of lecanoralean taxa represented in the data set.

The partition homogeneity test (PHT) of the combined data set as implemented in PAUP*4.0d64 indicated that the SSU and LSU data sets were congruent ($p = 0.02$) and therefore, combinable. Cunningham (1997) suggests that PHT p-values greater than $p = 0.01$ reflect congruent data sets, that if combined will either improve or will not negatively effect phylogenetic accuracy. Therefore, the combined data set was used in phylogenetic analyses. The combined data set included 1656 characters, of which 287 were potentially parsimony-informative. Maximum parsimony, weighted parsimony and maximum likelihood analyses of combined SSU and LSU rDNA sequence data yielded identical topologies with respect to the placement of the *Dibaeis* relative to *Baeomyces*.

Two equally most parsimonious trees of 950 steps were generated from a branch-and-bound search of the combined data set using maximum parsimony. Weighted parsimony analysis of the combined data set resulted in a single most parsimonious tree that did not differ from the maximum parsimony trees at any strongly supported nodes (data not shown). Log likelihoods were calculated for these three trees; the two most parsimonious trees plus the single weighted parsimony tree. The phylogeny presented in Figure 2.2 is the one maximum parsimony tree which had the best log likelihood. As in the SSU rDNA tree (Figure 2.1), this combined SSU-LSU rDNA phylogeny (Figure 2.2) reveals that *Dibaeis baeomyces* forms a monophyletic clade with *Icmadophila ericetorum* and *Siphula ceratites* and is not within the *Baeomyces* clade. Once again, bootstrap support for this relationship is excellent at 100. Likewise, the *Baeomyces* clade is well supported with a bootstrap value of 100. Congruent with the SSU rDNA phylogeny, *Evernia prunastri* groups with the *Icmadophila* clade and *Loxosporopsis corallifera* is a sister taxon to the *Baeomyces* clade, but no bootstrap support exists for either of these relationships.

To further test the hypothesis that *Dibaeis* is more closely related to *Icmadophila* than it is to *Baeomyces*, Kishino and Hasegawa (1989) and Templeton (1983) tests, as implemented in PAUP* under likelihood tree scores and parsimony tree scores respectively (Swofford, 1999), were performed on the combined data set (Table 2.2). The

FIGURE 2.1. Single most parsimonious tree of 422 steps inferred from partial nuclear SSU rDNA sequence data. The inferred phylogeny was generated with a branch-and-bound search of 134 parsimony-informative characters with maximum parsimony criteria. Bootstrap values greater than 50 generated from 1000 bootstrap replicates are shown above branches. CI = 0.754, HI = 0.246



null hypothesis (H_0), that *Dibaeis* and *Baeomyces* form a monophyletic clade, was tested by enforcing the topological constraint which placed *Dibaeis* within the *Baeomyces* clade. Two equally most parsimonious trees were generated under this constraint and compared to the unconstrained trees. All tests produced p-values of <0.0001 for the constrained trees (Table 2.2), indicating that the null hypothesis (H_0) must be rejected and that *Dibaeis* and *Baeomyces* are not members of the monophyletic taxon, Baeomycetaceae *sensu* Dumortier (1829).

The primary goal of this study was to test the morphology-based hypothesis of Gierl and Kalb (1993) that those *Baeomyces* species with rose-colored, or pink apothecia should be segregated into *Dibaeis* with those brown apotheciate species retained in *Baeomyces*. The molecular data generated and analyzed in this study clearly supports this hypothesis. In both SSU rDNA and combined SSU-LSU rDNA phylogenies, *Dibaeis baeomyces* was never placed within the *Baeomyces* clade (Figures 2.1 and 2.2). In fact, those *Baeomyces* species with brown ascocarps formed a well-supported monophyletic clade. *Dibaeis baeomyces* grouped with another pink-apotheciate lichen, *Icmadophila ericetorum* and thus also supports the placement of *Dibaeis* in the Icmadophilaceae (Rambold et al, 1993). The placement of *Siphula ceratites* within the Icmadophilaceae in both sets of analyses is noteworthy as the genus *Siphula* is sterile (Hafellner, 1988; Poelt, 1974) and is consistent with the results of Stenroos & DePriest (1998). However, the placement of *Siphula* within the Icmadophilaceae is unclear; in the SSU rDNA analysis it groups with *Dibaeis baeomyces*, but in the combined analyses it groups with *Icmadophila ericetorum*. This relationship will be addressed in the future with more appropriate taxon sampling and additional phylogenetic analyses (Platt and Spatafora, in review).

FIGURE 2.2 Best (-ln L) of two equally most parsimonious trees of 950 steps generated from maximum parsimony analysis employing a branch-and-bound search of 287 parsimony-informative characters from the combined nuclear SSU and LSU rDNA data set. Bootstrap values greater than 50%, generated using 1000 replicates from full heuristic search of parsimony-informative characters, are shown above branches. CI = 0.682, HI=0.318

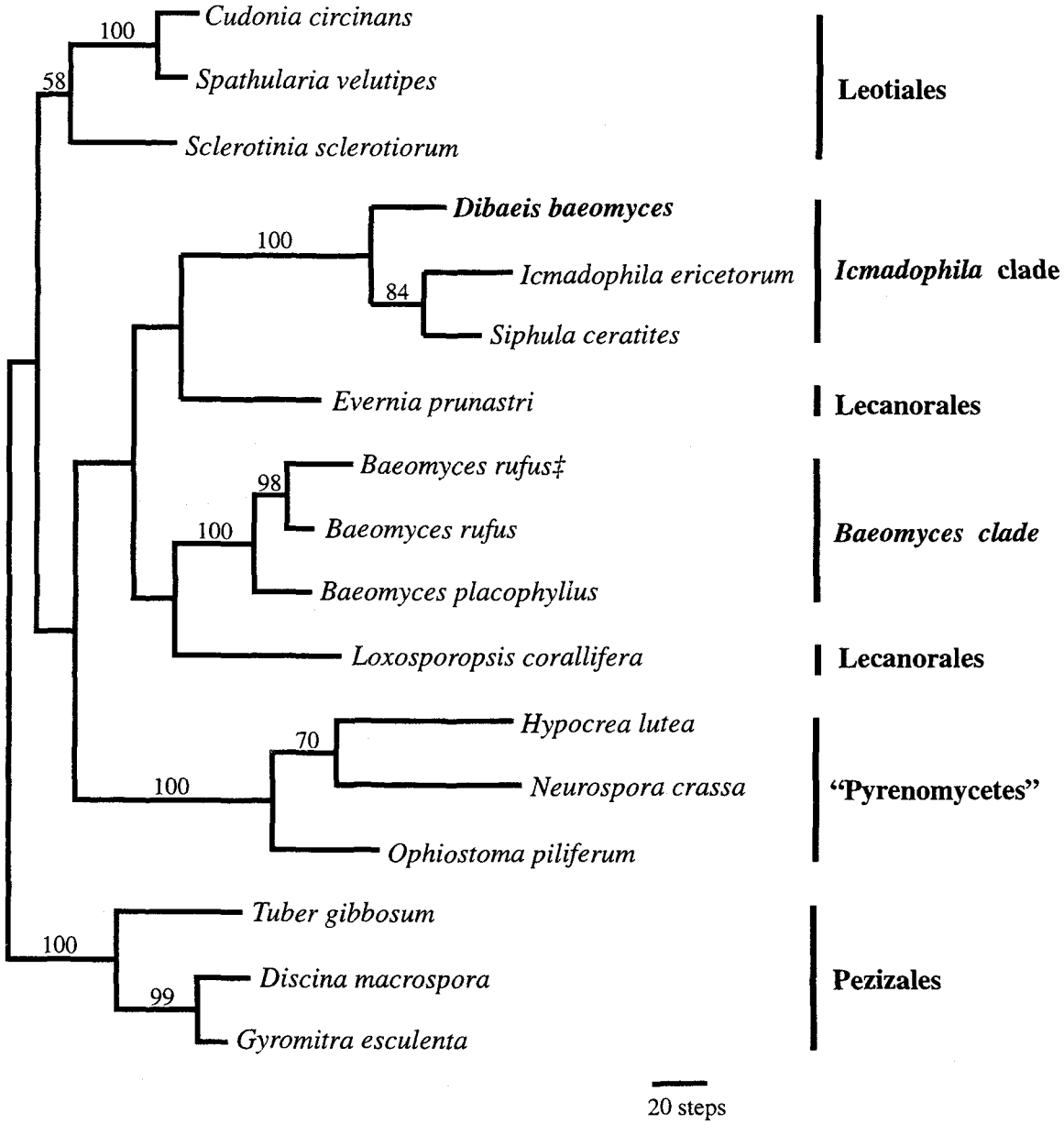


Table 2.2 Results of Kishino-Hasegawa and Templeton tests from the combined data set showing that with both tests, the null hypothesis of the constrained tree must be rejected.

	Unconstrained Trees		**Constrained Trees**	
	Tree 1	Tree 2	Tree 1	Tree 2
Length	950	950	1005	1005
-ln L	6957.51481	6959.24871	7165.00529	7163.79677
Kishino-Hasegawa	Best	p = 0.8863	p = <0.0001	p = <0.0001
Templeton	Best	p = 1.0	p = <0.0001	p = <0.0001

The current ordinal-level classification of the Ascomycota places both the Baeomycetaceae and Icmadophilaceae in the Helotiales (Hawksworth et al., 1995). The validity of this classification is unresolved in these analyses and in other molecular phylogenetic studies that included a large sampling of lecanoralean taxa (Stenroos and DePriest, 1998). The Helotiales are a large and heterogeneous order and the underrepresentation of its many lineages in phylogenetic analyses is only now beginning to be appreciated (Pfister, 1997). In our analyses neither the Baeomycetaceae nor the Icmadophilaceae was strongly supported as a close relative of the nonlichenized helotialean families Sclerotiniaceae or Geoglossaceae (i.e., *Cudonia* and *Spathularia*) (Figures 2.1 and 2.2). The SSU rDNA-inferred phylogeny presented by Stenroos & DePriest (1998) agrees with this finding and also fails to provide evidence for a close relationship between these lichen families and *Leotia lubrica*. These results challenge the hypotheses of Chadefaud (1960) and Honegger (1983) who predicted a close relationship between *Baeomyces* and *Leotia* on the basis of ascus similarities. However, discretion should be used in concluding that the Baeomycetaceae and Icmadophilaceae are not closely related to other members of the Helotiales given the complex and potentially non-monophyletic nature of the order (Pfister, 1997). Therefore in the context of the Helotiales, it is more appropriate that these

data be interpreted at familial and generic levels and inferences about ordinal placement be reserved until additional data provide well-supported evidence for higher-level classifications.

Weighted parsimony of the SSU rDNA data set had no effect on topology relative to the position of *Dibaeis* and *Baeomyces* but increased resolution for the combined data set. Increasing the number of characters resulted in less resolution and an increase in the amount of homoplasy. The additional 519 characters (153 parsimony-informative) from LSU rDNA added to the SSU rDNA data set to form the combined data set, resulted in a decrease of the consistency index (CI) from 0.754 to 0.682 which reflects an increase in homoplasy. This increase in homoplasy may account for the lack of resolution and increase in the number of most parsimonious trees from one to two.

This study is the first to present data that statistically support the segregation of *Dibaies* from *Baeomyces* (Table 2.2). By enforcing the constraint of *Dibaeis baeomyces* within the *Baeomyces* clade, this null hypothesis could be directly and statistically tested using the Kishino-Hasegawa (Kishino & Hasegawa, 1989) and Templeton tests (Templeton, 1983). By comparing the most parsimonious and best log likelihood trees under the constraint with the most parsimonious unconstrained trees, a relatively conservative approach was taken. Even with such conservation, the p-values suggested the null hypothesis must be rejected and *Dibaeis baeomyces* is not part of the *Baeomyces* clade (Table 2.2).

Although all analyses clearly support the segregation of *Dibaeis* from *Baeomyces*, the ordinal classification of these two lichen families within the Helotiales has yet to be supported with molecular systematic data. The taxon sampling used in this study is inadequate for addressing the ordinal relationship of these two lichen families. To address this, more lichenized taxa and additional members of the Helotiales will be sampled for both the SSU and LSU rDNA. Without a broader sampling it will also be difficult to form robust hypotheses regarding the evolution of the number of lichenization events. If either

the Baeomycetaceae or Icmadophilaceae is in the Helotiales *sensu stricto*, a unique gain of lichenization independent from that of the Lecanorales would be the favored hypothesis. If both of these families are finally resolved within the Helotiales but do not appear as sister taxa, then either a gain followed by a loss or two gains of lichenization within the order would be suggested, depending on topology. Although these data firmly support the separation of *Dibaeis* from *Baeomyces* and its inclusion in the Icmadophilaceae, many questions still remain, not the least of which is the ordinal placement of these two lichen families and the subsequent consequences on current concepts of the number of lichenization events within the Kingdom Fungi.

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CHAPTER 3

Evolutionary Relationships of Nonsexual Lichenized Fungi: Molecular Phylogenetic Hypotheses for the Genera *Siphula* and *Thamnolia* from SSU and LSU rDNA

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3.1 Abstract

The nonsexual lichenized family Siphulaceae is comprised of the obligately sterile genera, *Siphula* and *Thamnolia*. These genera are currently placed in the order Lecanorales, apparently based soley on the character of lichenization since apothecia and other lichen reproductive structures are absent. Using the nucleotide characters of rDNA, we tested the phylogenetic position of these genera among the Ascomycota. Cladistic analyses of SSU nrDNA and partial LSU nrDNA did not support the recognition of the Siphulaceae. Rather, *Siphula* and *Thamnolia* represent independent origins of nonsexual lichens among the Icmadophilaceae; a family which is currently classified in the order Helotiales. In addition to these molecular characters, the secondary chemistry of these genera also supports their affinity with the Icmadophilaceae. We propose taxonomically emending the Icmadophilaceae to include the genera *Siphula* and *Thamnolia*. We also discuss the phylogenetic relationships between the Icmadophilaceae and the Baeomycetaceae, the other family of lichenized fungi classified in the Helotiales. We provide cladistic evidence demonstrating that the Baeomycetaceae is a distinct taxon from the Icmadophilaceae and the two families represent independent lichenization events. We also discuss the phylogenetic relationships between these families and other higher taxa (e.g., families and orders) of the Ascomycota.

3.2 Introduction

Nearly half of all known ascomycetes are lichenized (Hawksworth and Hill, 1984), yet lichenology and mycology have traditionally been treated as distinct disciplines. This division has hindered phylogenetic studies of ascomycetes, and fungi in general. Evolution of the lichen habit from saprobic and parasitic fungi is recognized to have occurred multiple times, both within the Basidiomycota and Ascomycota (Gargas et al., 1995; Lutzoni and Vilgalys, 1995). In order to form complete and robust fungal phylogenetic hypotheses,

data from lichenized taxa must be integrated with that from nonlichenized taxa (Hawksworth, 1988). However, this integration has been recognized as difficult to obtain for some time. Dennis (1960) recognized the need for the integration of lichens with fungi but noted that "the complexity of both groups makes this a difficult goal to achieve." Traditional systematic methods based on morphological data may not always be possible, as many lichenized ascomycetes do not share the same morphological characters as their nonlichenized relatives. For example, many lichens have adopted a predominantly asexual habit and rarely produce ascocarps. The asexual propagules they produce, mainly isidia and soredia, are unique to lichens and are not produced in nonlichenized ascomycetes. A few lichens appear to be obligately sterile and have never been observed to produce ascocarps. This lack of cross-comparative morphology has impeded attempts to integrate lichenized and nonlichenized taxa on the basis of traditional systematic methods. Molecular characters, and specifically ribosomal DNA (rDNA), have a great advantage in that they are heritable, ubiquitously distributed across taxa, and independent of morphology. The use of rDNA nucleotide sequences in fungal systematics has been of great benefit in the phylogenetic classification of asexual fungi with respect to sexual taxa (Berbee and Taylor, 1993; Guadet et al., 1989; Ogawa et al., 1997; Rehner and Samuels, 1995; Rehner and Samuels, 1994). In addition, nuclear rDNA (nrDNA) sequence data has been useful for elucidating relationships between lichenized and nonlichenized fungi (Gargas et al., 1995; Lutzoni and Vilgalys, 1995). The above mentioned limitations of morphological data is a primary reason for uncertain phylogenetic placement of some asexual fungi and lichens in classifications based on sexual characters.

The Siphulaceae represents an example of lichenized fungi that have an uncertain taxonomic placement. This uncertainty is not only due to lichenization, but also stems from the fact that they are nonsexual. That is, members of this family not only fail to produce ascocarps but they also lack the specialized asexual propagules present in many lichens. Dahl and Krog (1973) have treated both *Siphula* and *Thamnolia* as members of the

Usneaceae, apparently based on gross thallus morphology. But Poelt (1974) first recognized the natural grouping of the obligately sterile genera *Siphula* and *Thamnolia*, along with *Endocena* and suggested they be considered a separate family, "the Siphulaceae", allied to the Cladoniaceae on the basis of chemistry. This provisional name, "Siphulaceae" was subsequently used by several authors (Dodge, 1973; Poelt and Vezda, 1981). However, the relationship of *Siphula* and *Thamnolia* to the Cladoniaceae has recently been demonstrated to be disparate on the basis of SSU nrDNA sequence data (Stenroos and DePriest, 1998). Because of the obligately sterile nature of this group, the Siphulaceae was later placed in the Deuteromycotina or imperfect fungi (Hafellner, 1988). However, abandonment of the Deuteromycotina (Kendrick, 1989) has led to placement of the Siphulaceae in the Lecanorales (Ascomycota), usually with a notation of *incertae sedis* (Hawksworth et al., 1995). This ordinal placement is apparently based primarily on the character of lichenization, because all sexual and asexual characters are lacking. The Siphulaceae is most commonly recognized as containing the genera *Siphula* and *Thamnolia*, however, Galloway (1985), as well as others (Dodge, 1973; Kantvilas, 1994) also recognize *Endocena* within this family. For purposes here, we follow the classification scheme in the Eighth edition of Dictionary of the Fungi (Hawksworth et al., 1995) and therefore only consider *Siphula* and *Thamnolia*.

The ecology of members of the Siphulaceae is varied. *Siphula* is a moderately-sized genus of approximately twenty-five species (Hawksworth et al., 1995). The genus generally has cosmopolitan distribution, but centers of species diversity are apparent, specifically in southern Africa (Mathey, 1974), South America, Hawaii (Magnusson and Zahlbruckner, 1945), and Tasmania and New Zealand (Galloway, 1985; Kantvilas, 1987). *Siphula* can be found associated with a wide range of substrates and habitats and is generally characterized by well-developed, root-like rhizines (Kantvilas, 1994). The thallus is generally foliose to fruticose and consists of a trebouxioid photobiont. The genus *Thamnolia*, which is also fruticose with a trebouxioid photobiont, consists of only two

species, or chemotypes; *T. vermicularis* and *T. subuliformis* (Asahina, 1937; Ozenda and Clauzade, 1970; Poelt, 1969). These two taxa are sometimes referred to as chemotypes because the difference in secondary chemistry is the only autapomorphic trait they possess (Asahina, 1937; Krog et al., 1980; Nimis, 1993; Purvis et al., 1992; Santesson, 1993) . *T. vermicularis* contains thamnolic acid, but *T. subuliformis* possesses baeomycesic and squamatic acids (Asahina, 1937). Although the distribution of *Thamnolia* is also cosmopolitan, it is generally limited to alpine and subalpine zones (Culberson, 1963; Galloway, 1985). Furthermore, this genus generally possesses a bipolar distribution pattern with respect to chemotypes. *T. subuliformis* is more common in the northern hemisphere, whereas *T. vermicularis* is more common in the southern hemisphere (Galloway, 1985; Sato, 1963); however, this bipolar distribution pattern is not abrupt, and considerable regions of geographic overlap exist (Sato, 1963). This pattern of geographic distribution, along with the nonsexual nature of the genus, has led to interesting debates regarding dispersalist or vicariance biogeography and the distribution of *Thamnolia* (Culberson, 1963; Sato, 1963; Sheard, 1977).

Although the Siphulaceae has been placed in the Lecanorales, preliminary molecular analyses questioned this classification and suggested a closer alliance with members of the Helotiales *sensu lato* (Platt and Spatafora, 1997; Platt and Spatafora, 1998; Spatafora et al., 1995) . The order Helotiales is comprised of inoperculate discomycetes which exhibit a broad spectrum of lifestyles ranging from mycorrhizal to saprobic to parasitic, sexual to asexual, and lichenized to free-living. Because this order is predominantly nonlichenized, the acquisition of the lichen habit is hypothesized to be a recently evolved strategy in comparison with predominantly lichenized orders (i.e., the Lecanorales), which may have gained the lichen symbiosis much earlier in their evolutionary history (Hawksworth, 1988; Tehler, 1983). Of the thirteen families currently recognized in the Helotiales *sensu lato* (Hawksworth et al., 1995) only two, the Baeomycetaceae and Icmadophilaceae, are lichenized. The Icmadophilaceae is a recently erected family (Rambold et al., 1993)

consisting of five lichen genera; *Dibaeis, Icmadophila, Knightiella, Pseudobaeomyces*, and *Siphulella*. Gierl and Kalb (1993) reintroduced the name *Dibaeis*, previously placed in synonomy with *Baeomyces* as a nomenclatural replacement for *Baeomyces* species with rose colored ascocarps. The Baeomycetaceae has been retained for those *Baeomyces* species with brownish ascocarps. Phylogenetic relationships of the remaining eleven helotialian families have remained unclear, as this complex order has been slow in attracting the attention of molecular systematists (Pfister, 1997).

To test and refine phylogenetic hypotheses for the evolution of nonsexual lichens of the Siphulaceae, we designed taxon sampling to include lichenized and nonlichenized fungi from the numerous groups of the Ascomycota. To test the phylogenetic utility of lichen acids at higher taxonomic levels, we sampled lichen species with secondary chemistry similar and dissimilar to the Siphulaceae. For example, members of the Icmadophilaceae were chosen due to the commonality of baeomycesic acid, and more generally depsides, with members of the Siphulaceae. Below we discuss phylogenetic hypotheses regarding the evolution of lichenization among the Ascomycota and potentially within the Helotiales, and the evolution of nonsexual lichenized fungi of the Siphulaceae. Furthermore, we provide evidence to support emendation the Icmadophilaceae (Leotiaeles) to include the genera *Siphula* and *Thamnolia*.

3.3 Materials and Methods

The nuclear SSU and LSU rDNA nucleotide sequences determined in this study were obtained from freshly collected specimens or herbarium material. Voucher specimens were deposited in the Oregon State University Mycological Collection (OSC). Additional sequences were retrieved from GenBank. The taxa sampled in this project are listed in Table 3.1.

Table 3.1 Species included in this study with GenBank accession numbers for complete nuclear SSU and partial nuclear LSU sequences.

Taxon	GenBank Accession Number	
	SSUrDNA	LSU rDNA
Amauroascus albicans	-	U17914
Aspergillus fumigatus	M60300	U28460
Athelia bombacina	M55638	-
Baeomyces heteromorphus (1)	-	AF113740[†]
Baeomyces heteromorphus (2)	-	AF113742[†]
Baeomyces placophyllus	AF113719[†]	AF107560[†]
Baeomyces rufus (1)	AF113717[†]	AF107558[†]
Baeomyces rufus (2)	AF113718[†]	AF113743[†]
Blastomyces dermatitidus	X59420	-
Blumeria graminis	L26253	-
Botryosphaeria ribis	U42477	-
Candida albicans	X53497	-
Capronia pilosella	U42473	-
Cladonia bellidiflora	U60900	-
Cochliobolus sativus	U42479	-
Cudonia circinans	-	AF107553[†]
Cudonia confusa	Z30240	-
Dibaeis absolutus	-	AF113731[†]
Dibaeis baeomyces (1)	AF113712[†]	AF113730[†]
Dibaeis baeomyces (2)	AF113713[†]	AF107555[†]
Dictyonema pavonia	U23541	-
Discina macrospora	-	U42678
Dothidea hippophaeos	U42475	-
Eurotium herbariorum	-	U29554
Evernia prunastri (1)	AF113721[†]	AF113745[†]
Evernia prunastri (2)	-	AF107562[†]
Geoglossum glabrum	-	AF113738[†]
Geoglossum nigritum	AF113716[†]	-
Gyromitra esculenta	U42648	U42675
Hypocrea lutea	-	U00739
Hypomyces chrysospermus	M89993	-
Icmadophila ericetorum (1)	AF113709[†]	AF107556[†]
Icmadophila ericetorum (2)	-	AF113728[†]
Icmadophila ericetorum (3)	-	AF113729[†]
Inermisia aggregata	Z30241	-
Lecanora dispersa	L37535	-
Leotia lubrica	L37536	-
Leotia viscosa	AF113715[†]	AF113737[†]
Leucostoma persoonii	M83259	-
Loxosporopsis corallifera	AF113722[†]	-
Morchella elata	U42641	-
Neolecta vitellina	Z27393	U42695
Neurospora crassa	X04971	-
Ophiostoma piliferum	-	U47837
Ophiostoma ulmi	M83261	-
Orbilia auricolor	U72598	-
Orbilia delicatula	U72603	-

Table 3.1 (Continued)

Peltigera neopolydactyla	X89218	-
Pertusaria saximontana	AF113720†	-
Pilophorus acicularis	U70960	-
Porpidia crustulata	L37735	-
Pyrenophora tritici-repentis	U42486	-
Saccharomyces cerevisiae	V01335	-
Sclerotinia sclerotiorum	L37541	Z73762
Sclerotinia veratri	-	AF113739†
Siphula ceratites (1)	U72712	AF107557†
Siphula ceratites (2)	-	AF113723†
Siphula coriacea	-	AF113724†
Siphula pickeringii	-	AF113727†
Siphula polyschides (1)	AF113710†	AF113725†
Siphula polyschides (2)	AF113711†	AF113726†
Spathularia flavida	Z30239	-
Spathularia velutipes (1)	-	AF113734†
Spathularia velutipes (2)	-	AF113735†
Spathularia velutipes (3)	-	AF113736†
Spongipellis unicolor	M59760	-
Stictis radiata†	-	AF113746†
Thamnolia subuliformis	AF113714†	AF113733†
Thamnolia vermicularis	-	AF113732†
Tuber gibbosum	U42663	U42690

†Sequences determined in this study for which source and voucher information is provided in GenBank accession.

3.3.1 DNA Extractions

All samples were first extracted with acetone for approximately 30-60 minutes; this extraction was repeated two to three times. Crude DNA extracts were prepared by a modified CTAB method (Gardes and Bruns, 1993). Unless otherwise indicated, all reagents were obtained from Sigma Chemical Company, St. Louis, MO. Samples were ground with liquid nitrogen immediately before the addition of 65°C 2X CTAB buffer supplemented with 2% β-mercaptoethanol. One phenol:chloroform (1:1) followed by two chloroform:isoamyl alcohol (24:1) extractions preceeded Isopropanol precipition of DNA. The final step was purification of the crude DNA extract, using the Elu-Quik DNA Purification Kit (Schleicher & Schuell, Inc., Keene, NH)

3.3.2 Polymerase Chain Reaction and DNA Sequencing

Polymerase chain reactions (PCR) were performed in 50 µl reaction volumes that contained approximately 50 ng of DNA, 10mM Tris-HCl, pH 8.3, 50mM KCl, 0.005% Tween 20, 0.005% NP-40, 1.5mM $MgCl_2$, 62.5 µM dNTP mix (containing equal amounts of each of the four deoxyribonucleotide triphosphates), 0.5 µM of each PCR primer, and 2.5 units Replitherm™ Thermostable DNA Polymerase. The genes that code for the Nuclear Small Subunit rDNA (SSU nrDNA) were amplified with primers NS17UCB - NS24UCB (Gargas and Taylor, 1992) [nu-SSU-0072-5' and nu-SSU-1750-3' respectively, following the nomenclature recently proposed by Gargas and Depriest (1996)]. PCR reactions were overlaid with mineral oil and placed in an MJ Research, Inc. PTC-100™ Programmable Thermal Controller and subjected to the following conditions: 94°C for 3 minute, 35 cycles of 94°C for 1 minute, 51°C for 30 seconds, and 72°C for 1 minute then 5 cycles of 94°C for 1 minute, 53°C for 30 seconds, then 72°C for 1 minute plus 5 seconds per cycle, and finally 72°C for 5 minutes.

LSU nrDNA PCR reactions utilized the primer pairs LROR and LR5 (Vilgalys and Sun, 1994). All reaction concentrations were identical to those used in the SSU reactions with the exception of final primer concentration which was 0.3 µM. The cycling conditions of PCR were: 94°C for 3 minutes, 39 cycles of 94°C for 1 minute, 48°C for 30 seconds, and 72°C for 45 seconds, and 3 minutes at 72°C.

PCR products were visualized on a 1% agarose gel stained with ethidium bromide and quantified using Gibco-BRL low DNA Mass™ Ladder. PCR products were purified by adding one half volume of 4.5 M NH4OAc was added to the PCR product, followed by

brief vortexing, then addition of two volumes isopropyl alcohol. This mixture was vortexed briefly and then allowed to precipitate at room temperature for 15 minutes. Each sample was then centrifuge at 10,000g and rinsed with 70% ethanol. The purified pellet was resuspended in a volume of doubled distilled water to give 20-25 ng/µl. The purified product was sequenced on an ABI Prism™ Model 377 (version 2.1.1) automated DNA sequencer (Perkin-Elmer) at the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University. Both the coding and template strands of PCR products were sequenced utilizing the sequencing primers NS2, SR7R, SR11R, NS4, NS5, and NS24 for SSU nrDNA and LR0R, LR3 for LSU nrDNA.

3.3.3 Taxon Sampling

Fourty-eight taxa representing 45 species are included in the complete SSU rDNA data set. Three taxa representing basidiomycete species were used as an outgroup. Fourty-two taxa representing 29 species are included in the partial LSU data set. *Neolecta vittelina* was used as the outgroup in LSU analyses. Thirteen complete SSU and 32 partial LSU sequences were determined in this study, the remainder were obtained from GenBank. Sequences used for the phylogenetic reconstructions in this study are listed in Table 3.1.

3.3.4 Sequence Alignment and Phylogenetic Analysis

All sequences determined in this study were edited manually, comparing the computerized sequence read to that shown on the color electropherogram. Introns were excluded from the SSU nrDNA data set. SSU nrDNA nucleotide sequences were aligned by visual, color-based estimation using SeqApp version 1.9a169 (Gilbert, 1992). Large subunit sequences were aligned initially using ClustalX (Thompson et al., 1997), final adjustments of the alignment were made manually in SeqApp (Gilbert, 1992). Ambiguous nucleotides were

coded using standard IUPAC nucleic acid nomenclature of n, r, k, etc. Indels were treated in one of two ways; as informative, denoted with an "-" (gap) or as missing data, denoted with a ".". Those positions with known nucleotides but which were ambiguously alignable with respect to bordering positions were treated as missing data. This method is based on the recoding methods of Bruns et al. (1992). Positions longer than four nucleotides with ambiguous alignment were excluded from analyses. Both the complete SSU and partial LSU rDNA alignments are available from the TREEBASE at http://herbaria.harvard.edu/treebase/.

Maximum parsimony analyses were performed using PAUP*4.0d57 (Swofford, 1997). A series of analyses were carried out to test the phylogenetic relationships between ingroup taxa. Heuristic searches utilizing tree-bisection reconnection and 100 random sequence additions were employed. In both data sets a transition:transversion (TS:TV) bias of approximately 1.6:1 was observed. Based on this observation, weighted parsimony analyses were performed using weights derived from the reciprocal of the TS:TV under the same search conditions as above.

Support for clade stability was estimated with bootstrapping (Hillis and Bull, 1993) and decay indices (Bremer, 1988). Bootstrap values were generated with 100 replicate-heuristic searches on all parsimony-informative characters using 5 random sequence addition replications and tree-bisection reconnection branch swapping algorithms in PAUP*. Decay indices were calculated with AutoDecay version 4.0 (Eriksson, 1998) with constraints tested in PAUP* and decay values output as a NEXUS tree file displayed with TreeView (Page, 1996).

In order to provide additional statistical support for inferred phylogenetic relationships in the SSU data set, a Kishino-Hasegawa test as implemented in PAUP* was performed. A total of 91 trees were scored using the Kishino-Hasegawa test; an abbreviated list of these trees and the results are given in Table 3.2. The trees tested fell into two categories; (i) unconstrained trees which were generated by parsimony analyses in

PAUP*, and (ii) constrained trees generated by parsimony analysis in PAUP* with topological constraints enforced. These latter trees were initially drawn from the default bush in MacClade 3.0 (Maddison and Maddison, 1992) to depict specific phylogenetic hypotheses (constrained trees). These trees, representing seven distinct phylogenetic hypotheses, were then subjected to a random sequence addition heuristic search of 50 replicates (TBR branch swapping) in PAUP* with topological constraints enforced under maximum parsimony criteria with gaps treated as missing and as newstate or under weighted parsimony criteria based on the observed ti/tv ration of 1.6:1 with gaps treated as missing data. Eleven of the unconstrained trees tested were generated using maximum parsimony criteria in PAUP* with gaps treated as missing or gaps treated as newstate. Six of the unconstrained trees were generated using weighted parsimony criteria based on the observed ti/tv ratio of 1.6:1 and gaps treated as missing data. Maximum Likelihood estimations were based on Felsenstein (1984) (Table 3.2) or Felsenstein (1981) models (data not shown). Starting branch lengths were obtained using Rogers-Swofford approximation method (Swofford, 1997) and a molecular clock was not enforced.

3.4 Results

3.4.1 SSU nrDNA

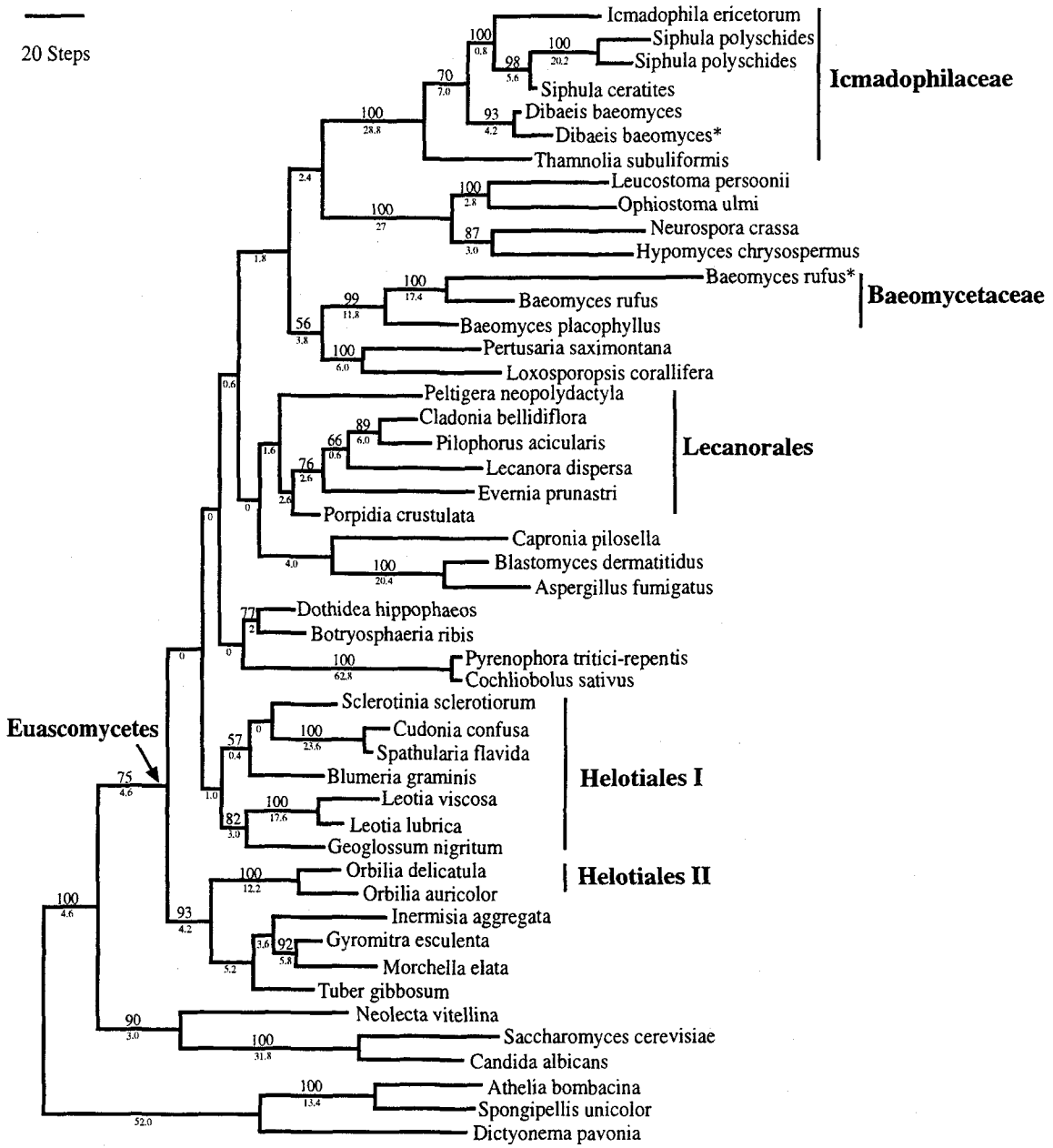
PCR products for the SSU rDNA ranged in size from approximately 1700bp to 2400bp (primer pair NS17-NS24). This size variation was attributed to the presence of inserts and/or introns common in ascomycete SSU nrDNA (Gargas et al., 1995). Phylogenetic analysis of the complete SSU nrDNA data set included 1743 characters for each of fourty-eight taxa. 478 of these characters were potentially parsimony-informative. Maximum parsimony analyses performed with gaps treated as missing data and as newstate, yielded eight and three equally most parsimonious trees, respectively (data not shown). All trees included the same basidiomycete outgroup designation.

In all trees, *Siphula* and *Thamnolia* formed a monophyletic clade with members of the Icmadophilaceae, i.e., *Icmadophila* and *Dibaies*. Support as measured by both bootstrap and Bremer support (decay indices) was excellent for this clade with values of 100 and 28.8, respectively (Figure 3.1). *Siphula* formed a well-supported, monophyletic clade that was a sister group to *Icmadophila ericetorum*. The two specimens of *Dibaeis baeomyces* sampled, one collected from the northern hemisphere and the other from the southern hemisphere, formed a monophyletic group with strong support within the Icmadophilaceae. *Thamnolia subuliformis* was also a well-supported member of the Icmadophilaceae and did not group most closely with species of *Siphula*.

Two additional clades comprised solely of lichen-forming species are also present in the weighted parsimony analysis (Figure 3.1). One clade includes species of *Baeomyces* and other lichen-forming fungi. The species of *Baeomyces* formed a monophyletic clade with bootstrap value of 99 and a decay index of 11.8. This Baeomycetaceae clade grouped with the lichenized species *Pertusaria saximontana* and *Loxosporopsis corallifera*. These latter two species formed a well-supported pair, but their grouping with *Baeomyces* was not strongly supported by the data. The "Lecanorales clade" also comprised solely of lichen-forming members, is monophyletic although not supported with bootstrap. In addition to typical lecanoralean taxa, this clade includes *Peltigera neopolydactyla* which is typically classified in the Peltigerales rather than the Lecanorales.

Two Helotiales clades also appear in all SSU rDNA analyses. The "Helotiales I" clade is paraphyletic due to the position of *Blumeria graminis*. This clade lacks bootstrap support and has a decay index of 1.0. *Cudonia confusa* and *Spathularia flavida* are well-supported sister species with a bootstrap value of 100. There is no support at the node joining *Cudonia* and *Spathularia* with *Sclerotinia sclerotiorum*. The position of *Blumeria gramimis* has minimal bootstrap support of 57 and a low decay index of 0.4. The relationships within and between *Leotia* and *Geoglossum nigritum* have bootstrap support

Figure 3.1 Best (-ln likelihood) of six equally most parsimonious trees of 2407.4 steps inferred from weighted parsimony analysis of complete SSU nrDNA. CI = 0.529, HI = 0.471, RI = 0.580 and RC = 0.307. Numbers above branches are bootstrap values from 100 replicate-heuristic searches on all parsimony-informative characters using 5 random sequence addition replications and tree-bisection reconnection branch swapping algorithms in PAUP*. Decay indices (Bremer support values) are shown below branches.



of 100 and 82, respectively. The "Helotiales II" clade places *Orbilia* as a sister group to the Pezizales with strong bootstrap support of 93 and Bremer support of 12.2.

The results of Kishino-Hasegawa tests (Kishino and Hasegawa, 1989) are shown in Table 3.2. Eight-hundred ninety-three distinct data patterns were detected under each maximum likelihood estimation model implemented (F81 and F84). Identical trees were also rejected under both models. P-values of <0.0001 were calculated for all trees under the constraint of the Siphulaceae in the Lecanorales clade (Hypothesis V). Additional significant p-values (significant at $P < 0.05$) were calculated for 24 other trees; 22 trees representing various phylogenetic hypotheses for the Helotiales (Hypotheses I & II) and two trees representing a monophyletic Siphulaceae (Hypothesis VI).

3.4.2 LSU nrDNA

LSU nrDNA amplicons of approximately 900bp were produced using the primer pair LR0R-LR5. To avoid problems associated with ambiguous positional homology, the divergent domains (D1 and D2) of the LSU data set were excluded from analyses.

Two equally parsimonious trees of 745 steps (CI = 0.530, HI = 0.470, RI = 0.744, RC = 0.394) were generated using the branch and bound search option of PAUP*. 557 characters were included with 163 of these being parsimony-informative. Figure 3.2 shows that *Siphula* is once again, placed within the Icmadophilaceae clade with bootstrap support of 99 percent. Most species of *Siphula* formed a sister-group to *Icmadophila ericetorum* with bootstrap support of 99, however, *Siphula coriacea* is sister to *Dibaeis* with bootstrap support of 64. This is the only species of *Siphula* sampled that fell outside of the main *Siphula* clade. *Dibaeis* formed a monophyletic clade with bootstrap support of 84. *Thamnolia* is included in this Icmadophilaceae clade with bootstrap support of 100. The absolute genetic distance between *Thamnolia subuliformis* and *Thamnolia vermicularis* was 33 (0.065 mean distance adjusted for missing data), which is greater than that of any of the other genera of different species within the Icmadophilaceae clade. For comparison,

Table 3.2 Results of Kishino-Hasegawa tests under the F84 model and implemented in PAUP* using empirical nucleotide frequencies. Criteria were either maximum parsimony (mp) or weighted parsimony (wp) based on the observed ti/tv ratio. Values in **bold** indicate significant p-values as determined by the two-tailed test. Hypothesis I: The Helotiales and Siphulaceae constrained to form a monophyletic clade. Hypothesis II: The Helotiales (excluding *Orbilia*) and the Siphulaceae constrained to form a monophyletic clade. Hypothesis III: The "Icmadophilaceae" (including *Siphula* and *Thamnolia*) constrained to a single monphyletic clade with other Helotiales (excluding *Orbilia* and *Baeomyces*). Hypothesis IV: The "Icmadophilaceae" (including *Siphula* and *Thamnolia*) and Leccanorales constrained to form a monophyletic clade. Hypothesis V: The Siphulaceae and Lecanorales constrained to a single monophyletic clade. Hypothesis VI: The Siphulaceae constrained to monophyly. Hypothesis VII: All lichenized taxa constrained to monophyly.

Topology	# Trees	Criterion	Gaps	Range -lnL	Range P*
unconstrained	8	mp	missing	13880.64110 - 13886.31250	0.3952– 0.5184
	3	mp	newstate	13882.74243 - 13935.77491	0.0568 – 0.4633
	6	wp	missing	13868.44800 – 13869.94571	0.4107 – Best
Hypothesis I	3	mp	missing	14011.33873 – 14023.80731	**0.0003 – 0.0009**
	15	mp	newstate	13995.53936 – 14018.85189	**0.0004 – 0.0011**
	1	wp	missing	14018.01835	**0.0001**
Hypothesis II	1	mp	missing	13964.64496	**0.0066**
	1	mp	newstate	13969.23738	**0.0090**
	1	wp	missing	13956.44308	**0.0069**
Hypothesis III	2	mp	missing	13913.62100 – 13914.64575	0.1301 – 0.1682
	1	mp	newstate	13922.45879	0.1227
	1	wp	missing	13909.12746	0.1915
Hypothesis IV	6	mp	missing	13906.53882 – 13922.77842	0.0600 – 0.1606
	4	mp	newstate	13904.19426 – 13906.53882	0.1305 – 0.1686
	2	wp	missing	13904.92186 – 13905.11204	0.1024 – 0.1375
Hypothesis V	8	mp	missing	14111.55076 – 14113.30049	**<0.0001**
	4	mp	newstate	14109.20985 – 14111.62644	**<0.0001**
	6	wp	missing	14101.44025 – 14112.59019	**<0.0001**
Hypothesis VI	2	mp	missing	13905.77562 – 13905.97792	0.1474 – 0.1777
	1	mp	newstate	13911.38131	0.1358
	2	wp	missing	13900.07981	**0.0117**
	2			13900.27325	**0.0390**
Hypothesis VII	2	mp	missing	13889.44036 – 13889.64267	0.3243 – 0.3611

Table 3.2 (Continued)

5	mp	newstate	13897.61991 – 13924.73423	0.0938 – 0.2425
6	wp	missing	13881.35022 – 13881.54047	0.4549 – 0.5021

* = probability of getting a more extreme T-value under the null hypothesis of no difference between the two trees (two-tailed test) with significance at $P < 0.05$.

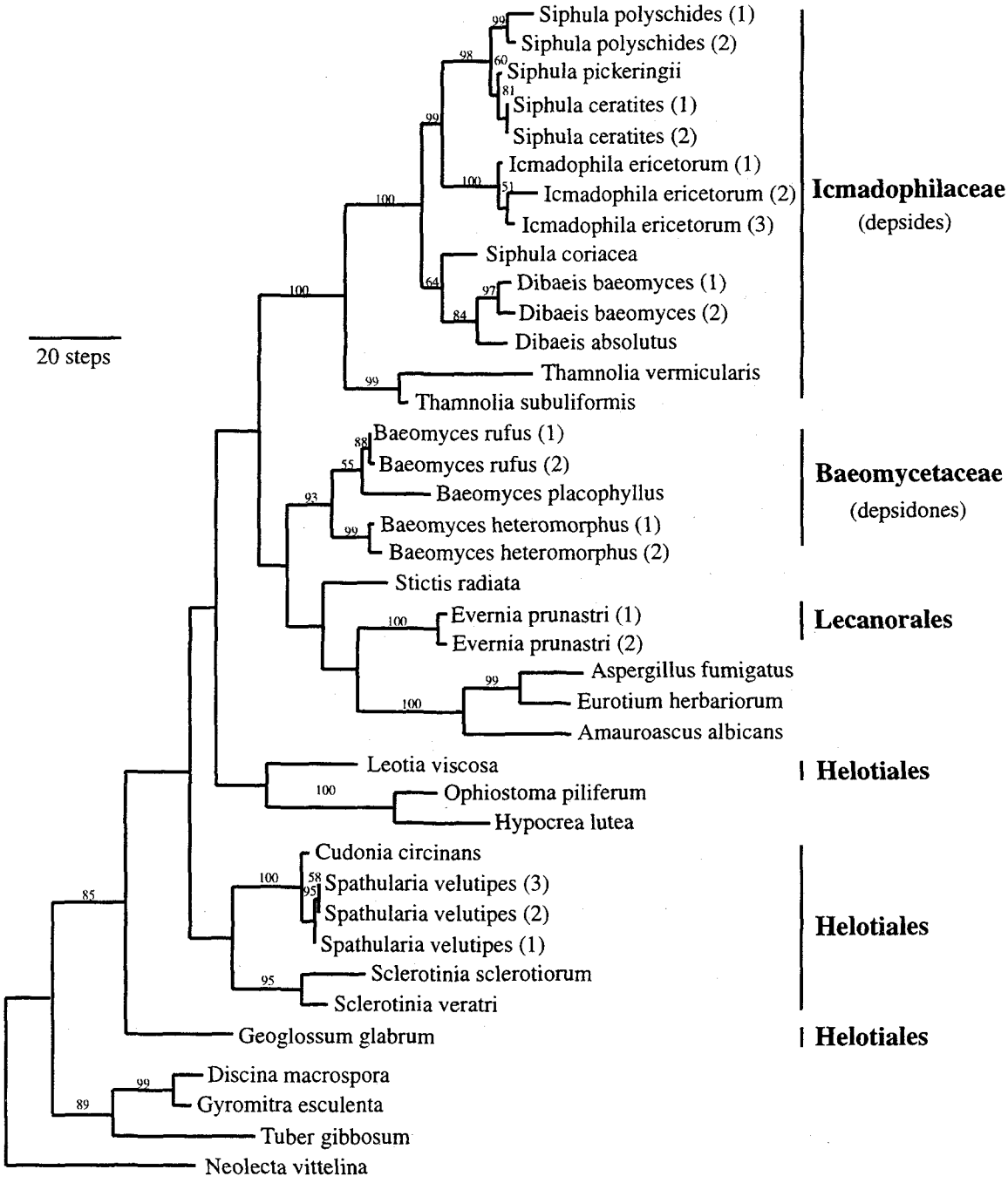
the greatest genetic distance within *Icmadophila ericetorum* was 9 (0.02 mean) and the absolute genetic distance between *Icmadophila ericetorum* and *Siphula ceratites* was 29 (0.057 mean value). The absolute genetic distance within *Dibaeis baeomyces* was measured at 7 (0.013 mean).

All species of *Baeomyces* form a monophyletic clade with bootstrap support of 93. *Evernia prunastri* is the only representative of the Lecanorales included in the LSU analysis and although it is placed sister to the plectomycetes there is no support for this relationship. Again, the Helotiales is not monophyletic with members appearing in three distinct regions of the cladogram. *Leotia viscosa* is sister to the pyrenomycetes, *Ophiostoma piliferum* and *Hypocrea lutea* but there is no bootstrap support for this relationship. *Cudonia, Spathularia* and *Sclerotinia* comprise a clade separate from both *Leotia viscosa* and *Geoglossum glabrum*. *Cudonia* and *Spathularia* are again sister with bootstrap support of 100. *Geoglossum glabrum* occupies a basal position within the ingroup topology with the outgroup node rooting between it and the rest of the ingroup.

3.5 Discussion

The molecular data presented here offer significant and robust evidence for the emendation of the Icmadophilaceae to include the provisionally named, nonsexual lichenized family Siphulaceae. Both SSU nrDNA (Figure 3.1) and LSU nrDNA (Figure 3.2) strongly support a monophyletic clade comprised of *Icmadophila, Dibaeis, Siphula,* and

Figure 3.2 One of two equally most parsimonious tress of 745 steps found by a branch-and-bound search of 557 included characters. (CI = 0.530, HI = 0.470, RI = 0.744, RC = 0.394) Bootstrap values from 100 replicate-heuristic searches using 5 random sequence addition replications and tree-bisection reconnection branch swapping algorithms are shown above branches. Numbers in parentheses at termini correspond to the sequence number from Table 1.



Thamnolia. Bootstrap support for these relationships, and the clade in general, is quite high. In both analyses the Icmadophilaceae clade is supported by bootstrap values and decay indices (Figure 3.1 and Figure 3.2). This finding concurs with that of Stenroos and DePriest (1998) who discuss the relationship of *Siphula* and *Thamnolia* with *Dibaeis* based soley on SSU rDNA data. They discuss the need for sampling additional members of the Icmadophilaceae and specifically *Icmadophila*. The study presented here not only includes the type species for the family Icmadophilaceae, *Icmadophila ericetorum*, but also takes a broader sampling approach with respect to the genus *Siphula* . In addition, we have provided statistical support for this hypothesis using the Kishino-Hasegawa test and included phylogenetic analysis of another gene region, the LSU rDNA. Kishino-Hasegawa maximum likelihood ratio test results of the SSU data set reject the null hypothesis that the Siphulaceae is a member of the Lecanorales ($P < 0.0001$) and disparate from the traditional members of the Icmadophilaceae (Table 3.2, Hypothesis V).

Emendation of the Icamadophilaceae, integrating *Siphula* and *Thamnolia* is proposed, rather than recognition of the Siphulaceae for several reasons. First, the "Siphulaceae" is not a distinct, monophyletic taxon. *Siphula* and *Thamnolia* do not appear as sister taxa in any of the phylogenetic analyses in this study. In fact, the position of *Siphula* and *Thamnolia* suggests the first divergence within the Icmadophilaceae clade was that of the lineages leading to these two genera (Figure 3.1 and Figure 3.2). In addition, results of Kishino-Hasegawa tests reject the null hypothesis ($P = < 0.05$) of a monophyletic Siphulaceae under the criteria of weighted parsimony (Table 3.2, Hypothesis VI). Finally, even the genus *Siphula* is not monophyletic (Figure 3.2). While *S. polyschides, S. pickeringii,* and *S. ceratites* are sister to *Icmadophila ericetorum* with boostrap support of 99, *S. coriacea* is sister to *Dibaies*. The nonmonophyly of *Siphula* was also discussed by Stenroos and DePriest (1998). They reported that a partial SSU nrDNA sequence of *S. coriacea* did not group with *S. ceratites* but instead had affinity for *Stereocaulon*. Our data do not support such a disparate relationship, and maintain *Siphula*

coriaceaa within the Icmadophilaceae. Figure 3.2 shows that all taxa sampled from the Siphulaceae are well-supported members of the Icmadophilaceae. As also mentioned by Stenroos and DePriest (1998), secondary chemistry leads us to question the monophyly of this nonsexual lichenized genus (Santesson, 1967). Although the Icmadophilaceae (Rambold et al., 1993) was introduced twenty years after the name "Siphulaceae" was used (Poelt 1974), the "Siphulaceae" was only provisional and therefore nomenclatural priority does not apply (Greuter et al 1994).

Another taxonomic concept addressed in our study is the number of *Thamnolia* species recognized. Many authors recognize *Thamnolia* as a monotypic genus (Krog et al 1980, Purvis et al 1992, Nimis 1993, Santesson 1993, Hawksworth et al 1995, Stenroos and DePriest 1998). Within this taxonomic concept, two or more subspecies are often recognized (Karnefelt and Thell 1995). However, other authors recognize two species (Asahina 1937, Poelt 1969, Ozenda and Clauzade 1970, Galloway 1985). In the LSU nrDNA cladogram we have considered *Thamnolia* to be comprised of (at least) two species, *Thamnolia vermicularis* and *Thamnolia subuliformis* (Figure 3.2). Our species recognition is based on the genetic distance measured within *Thamnolia* for the two taxa sampled. The two LSU sequences from *Thamnolia* determined in this study were from different chemotypes within the same geographic region (western North America). This absolute genetic distance was measured at 33 (0.065 mean) which is greater than the genetic distance measure within *Dibaies beoamyces* collected from northern versus southern hemisphere (7/0.013) and even greater than the genetic distance between *Icmadophila ericetorum* and *Siphula ceratites* (29/0.057) (data not shown). Furthermore, it is greater than the genetic distance within another nonsexual species, *Siphula polyschides* (8/0.016). While *Thamnolia vermicularis,* which contains thamnolic acid, is predominant in the southern hemisphere and *Thamnolia subuliformis*, which possesses squamatic and baeomycesic acids, is predominant in the northern hemisphere, broad areas of geographic overlap do exist. One such region where both chemodemes can be found is western North America;

from which the specimens in this study were collected. This chemodeme distribution has led to interesting hypotheses regarding vicariance versus dispersalist biogeography (Sheard 1997, Culberson 1963, Sato 1963, 1965, 1968). The nonsexual nature of this genus complicates this debate. Because *Thamnolia* apparently produces no specialized dispersal spores, thallus fragmentation is thought to be its only means of reproduction. Therefore, long-distance dispersal seems unlikely, and vicariance biogeography theory may explain its current patterns better than dispersalist biogeography theory.

In addition to providing support for revising current classification concepts, these data also test hypotheses regarding the evolution of nonsexual lichenized fungi. Understanding the phylogenetic relationships between lichenized and nonlichenized fungi has been a goal brought to the forefront of both mycology and lichenology in recent years. Another goal in fungal systematics has been to form robust phylogenetic hypotheses about the relationships between sexual and asexual fungi. This study has attempted to accomplish both goals, using nrDNA sequence data to form hypotheses regarding the relationships not only of lichenized and nonlichenized fungi, but also of sexual and asexual taxa. Assuming an obligately sterile state for *Siphula* and *Thamnolia*, both the SSU and LSU nrDNA data presented here support the hypothesis that sexual reproduction has been lost multiple times in the lichen family Icmadophilaceae. Cladistic analysis of the SSU nrDNA data set demonstrates loss of sexual reproduction once in the lineage leading to *Thamnolia subuliformis* and a second time in the lineage leading to *Siphula* (Figure 3.1). Furthermore, Kishino-Hasegawa maximum likelihood ratio test results of the SSU nrDNA data set suggest we must reject a single loss of sexual reproduction (monophyly of the Siphulaceae) under weighted parsimony criteria ($P = <0.05$) (Table 3.2, Hypothesis VI). The LSU nrDNA data also suggests more than a single loss of sexual reproduction within the Icmadophilaceae. A total of three losses of sexual reproduction are hypothesized in the LSU cladogram; one loss leading to the extant taxa of *Thamnolia*, another loss leading to the *Siphula* taxa which are sister to *Icmadophila ericetorum*, and a third loss, leading to

Siphula coriacea (Figure 3.2). A potential caveat to this hypothesis is that one or both of these nonsexual genera are not truly obligately sterile. It may be possible that very infrequent events of ascocarp formation, and thus sexual reproduction, do occur but so rarely that these events have yet to be observed.

This pattern of distribution of sexual and nonsexual species within the Icmadophilaceae clade is similar to patterns observed with anamorphic and teleomorphic nonlichenized fungal genera (Berbee and Taylor 1993, Rehner and Samuels 1994, Seifert et al 1995, Kuhls et al 1997). Although the holomorph concept is not typically used in lichenology, it may have legitimate application in the context of sexual and nonsexual lichens. Perhaps *Siphula* and *Thamnolia* are simply anamorphic species (holomorphs) which are lichenized. We use the term "anamorph", not as strictly mitosporic, but rather as defined by Korf and Hennebert (1993) to include the asexual (imperfect) form that is characterized only by presence or absence of conidia. In this case, *Siphula* and *Thamnolia* fit the latter criterion of no sexual reproduction and absence of conidia. For example, *Siphula ceratites* is probably not the anamorph of *Icmadophila ericetorum* but it is a closely related species, that happens to be anamorphic. These data lead us to predict that it is also probable that some of the "teleomorph" genera in the Icmadophilaceae (*Knightiella, Siphullela, Pseudobaeomyces*) which we have not yet sampled, are closely related to *Thamnolia*.

The data we present also demonstrate the nonmonophyly of the baeomycetoid morphology. This morphology is generally considered to consist of shortly stipitate apothecia produced on specialized thalline branches arising from a crustose or squamulose thallus. In both analyses, species with this general morphology fall into two separate clades which are also comprised of lichens with nonbaeomycetoid morphologies. The SSU cladogram (Figure 3.1) shows baeomycetoid morphologies fall within both the Icmadophilaceae and the Baeomycetaceae clades. Likewise, the LSU nrDNA data resolve baeomycetoid morphologies into the same two distinct clades (Figure 3.2). These

topologies also support the segregation of *Dibaeis* from *Baeomyces* (Gierl and Kalb 1993; Platt and Spatafora, 1999) and the inclusion of *Dibaeis* in the Icmadophilaceae (Rambold et al 1993). In doing so they reject the hypothesis that *Baeomyces* and *Icmadophila* both belong to the Baeomycetaceae (Tehler 1996). These data support the SSU rDNA hypotheses presented by Stenroos and DePriest (1998) which suggest that *Baeomyces rufus* and *Dibaeis baeomyces* are "phylogenetically distant". Our data demonstrates this disparate relationship, not only with cladistic analyses of SSU and LSU nrDNA, but also with maximum likelihood criteria of the Kishino-Hasegawa test. The results of Kishino-Hasegawa tests yield a p-value of < 0.05 for trees in which the Baeomycetaceae and Icmadophilaceae are constrained to monophyly (Table 3.2, Hypotheses I & II), suggesting we must reject the hypothesis that taxa with a baeomycetoid morphology are monophyletic. In fact, this constraint was conservative in allowing the inclusion of *Siphula* and *Thamnolia* and yet still results in rejection of the null hypothesis. Therefore, we must also reject the hypothesis that the baeomycetoid morphology is paraphyletic with members of the "Siphulaceae". In other words, we must reject a single origin of a baeomycetoid morphology with loss of this character in the lineages of *Siphula* and *Thamnolia.*

Secondary chemistry is also consistent with these nrDNA data which support independent origins of the baeomycetoid morphology. Members of the Icmadophilaceae (Figure 3.1 and 3.2) all possess the depside class of secondary compounds while members of the Baeomycetaceae produce lichen acids of the depsidone class (Figure 3.2). Secondary chemistry has traditionally been used in lichen systematics, but because characteristic lichen acids are usually absent from non-lichenized taxa, chemical characters have not been useful for integrating nonlichenized fungi. Although the taxonomic value of secondary products is not clear and these products are known to vary both quantitatively and qualitatively at various levels (Culberson et al 1988, Stenroos and DePriest 1998), it does not mean that these characters are not useful. The question remains, on what taxonomic level is secondary chemistry useful? Data from lichen acids has been used

frequently to separate species of the same genus but seems to be used less frequently at higher taxonomic levels. Figure 2 shows that possession of the depside class of lichen acids unite the Icmadophilaceae clade while the Baeomycetaceae clade members possess depsidones. Thus, under this sampling scheme lichens exhibiting a baeomycetoid morphology can be divided on the basis of the class of their secondary compounds.

Traditionally, the Baeomycetaceae, Icmadophilaceae, and Siphulaceae have all been placed in the Lecanorales (Henssen and Jahns 1973, Poelt 1974, Hale 1983). Recently however, the Baeomycetaceae and Icmdophilaceae were accepted as members of the Helotiales (Gierl and Kalb 1993, Rambold et al 1993, Tehler 1996, Hawksworth et al 1995) primarily based on similarities of ascus structure with *Leotia* (Chadefaud 1960, Honegger 1983). Molecular studies of SSU nrDNA have questioned the placement of either the Baeomycetaceae or the Icmadophilaceae in the Helotiales. One molecular phylogenetic study suggests that *Baeomyces* and *Icmadophila* have affinity for the Lecanorales but does not dispute their placement in the Helotiales (Eriksson and Hawksworth 1996). Stenroos and DePriest (1998) include a larger sampling of Lecanorales and while they acknowledge lack of resolution for ordinal placement they conclude that neither family belongs to the Lecanorales or Helotiales.

A significant stumbling block associated with all published molecular systematic studies of the Euascomycetes is the poor sampling of helotialean taxa (Pfister, 1997). This poor sampling has resulted in an artifactual monophyletic Helotiales (Gargas et al, 1995, Stenroos and DePriest, 1998; Spatafora, 1995) that renders the higher taxonomic placement of numerous lineages (e.g., Icmadophilaceae) problematical. The SSU nrDNA phylogram (Figure 3.1) highlights the major obstacle of forming robust hypotheses regarding ordinal placement of these families and assuming a monophyletic Helotiales. In our SSU analysis, the Helotiales is polyphyletic with two distinct Helotiales clades. The "Helotiales I" clade is paraphyletic due to the position of *Blumeria graminis*. Furthermore, there is no bootstrap support at this node and the decay index indicates the clade collapses with just a

single step (equivalent to a single transition). The Helotiales II clade is a well-supported sistergroup to the Pezizales with bootstap support of 93 and a decay index of 12.2. This provides strong evidence that some members of the Helotiales (i.e., *Orbilia*) are more closely related to the Pezizales than they are to other members of the Helotiales. These two species of *Orbilia* have not been included in previous SSU rDNA cladistic studies that have included some helotialean taxa within a broad taxon sampling (Gargas et al, 1995, Stenroos and DePriest, 1998; Spatafora, 1995) and this exclusion alone may contribute to the artifactual monophylly of the Helotiales. In addition, this taxon sampling does not include many Helotiales that may further demonstrate the polyphyletic nature of this large order.

These data reject the placement of the Icmadophilaceae in the Lecanorales, but also do not either support or reject the classification of the family in the Helotiales. In order to statistically test hypotheses of ordinal placement of the Icmadophilaceae, we implemented the Kishino-Hasegawa test on several trees generated under different parsimony criteria (Table 3.2). Results of these tests reveal that we must reject the hypothesis of a monophyletic Helotiales (including Baeomycetaceae and Icmadophilaceae) (Hypothesis I, P = < 0.05). When we relax the constraints to allow the most parsimonious placement of *Orbilia* (Hypothesis II), again the Kishino-Hasegawa test yeilds a p-value of < 0.05 suggesting we must reject the hypothesis of Baeomycetaceae and Icmadophilaceae (including the Siphulaceae) included in a monophyletic Helotiales. However, constraining only the Icmadophilaceae (and Siphulaceae) to the Helotiales and allowing the most parsimonious placement of the Baeomycetaceae and *Orbilia* (Hypothesis III) results in a p-value of >> 0.05 suggesting we cannot reject the hypothesis that the Icmadophilaceae is in the Helotiales.

The complexity of the euascomycete radiation, as well as the rate and number of informative characters present in SSU nrDNA leave molecular phylogeneticists with the difficult challenge of resolving the ascomycete "backbone" (Spatafora 1995, Berbee 1996). In some cases, resolving the ordinal placement of certain taxa may require being able to

resolve the euascomycete backbone; the ordinal placement of the Icmadophilaceae, and Baeomycetaceae may represent prime examples of this current phylogenetic impasse.

In pursuit of ulitmately defining a monophyletic Helotiales, the fact that *Orbilia* falls outside of the Helotiales in our analyses of the complete SSU rDNA (Figure 1) is also a noteworthy result. Although there is apparently evidence for maintaining the Orbiliaceae within the Helotiales (Pfister 1997), our analyses do not support its inclusion in the order. Our SSU nrDNA analyses and Kishino-Hasegawa test results suggest that *Orbilia* is a distinct taxonomic unit relative to the other helotialean taxa sampled. If future taxon sampling and phylogenetic analyses confirm this result, the Orbiliaceae may best be accomodated as unique order of Euascomycetes.

There are still many problems remaining with regard to the evolution of nonsexual lichenized fungi, their ordinal placement within the Euascomycetes and the systematics of the Helotiales. Our findings that the "Siphulaceae" is not a distinct taxon and that *Siphula* and *Thamnolia* were independently derived within the Icmadophilaceae represent progress towards understanding the evolution of lichens and nonsexual fungi. However, many questions remain, especially about the potential of gains and losses of sexual reproduction among lichenized fungi. Inclusion of additional meiotic taxa in future phylogenetic analyses may change current hypotheses and may better explain the distribution of character state differences. In addition, moving from a single gene phylogeny (SSU and LSU) towards an organismal phylogeny with the use of multiple loci in phylogenetic analyses may further our understanding of this fascinating group of fungi.

3.6 Acknowledgements

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CHAPTER 4

Exploration of Fungal Intragenic Spatial Phylogenetic Variation in Maximum Parsimony Analyses

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4.1 Abstract

Intragenic spatial phylogenetic variation (SPV) can arise from a variety of mechanisms, both natural and PCR- or method-mediated. While reconstructing fungal phylogenies based on nucleotide sequence data, we encountered a nuclear ribosomal SSU rDNA sequence with intragenic SPV. While PCR-mediated chimeric 16S sequences from microbial samples have been reported to occur at a fairly high rate, this is the first report of a potentially chimeric SSU rDNA sequence from a fungus. Because of the predominance of SSU rDNA sequence data for inferring fungal phylogenies, our goal in this study is to understand the impact of such sequences in phylogenetic data. We utilized a contrived chimera to evaluate the effect of such intragenic spatially phylogenetic variable sequences in maximum parsimony analyses. We also used selective removal of taxa and characters in phylogenetic analyses of SSU and LSU nrDNA to demonstrate topological effects of sequences with intragenic SPV. In these analyses, the partition homogeneity test was not effective in detecting incongruence between data partitions due to the presence of sequence data with intragenic SPV.

4.2 Introduction

Spatial phylogenetic variation (SPV) in sequence data can be extremely problematic to molecular phylogenetic studies, especially those based on explicit models of evolution (Grassly and Holmes, 1997) . Such systematic error can result in inaccurate and even grossly misleading phylogenies. Two primary types of SPV have been demonstrated in phylogenetic reconstruction; intergenic (Huelsenbeck and Bull, 1996) and intragenic

(Dorit and Ayala, 1995) SPV. The impact of intragenic SPV can be especially negative on phylogenetic reconstructions based on single genes (i.e., single gene phylogenies). However, the degree of accuracy and effects of such sequences are compounded by a myriad of other factors which include, but are not necessarily limited to, taxon-sampling, number of characters, and analytical method used.

Several natural phenomena can lead to intragenic SPV; these include various selective forces (Gritsun et al., 1995), functional constraints (Dorit and Ayala, 1995) , or recombination (Roberston et al., 1995; Bollyky et al., 1996). In addition SPV may occur as a result of PCR-mediated chimera formation (or recombination), especially when amplifying targets with highly conserved regions from community samples (Liesack et al., 1991). In fact, PCR-mediated chimeric SSU rDNA sequences have occurred at 32% frequency after 30 cycles of PCR amplification from mixed DNA populations (Wang and Wang, 1997) . Because PCR and parsimony analyses are frequently used in conjunction to both assess fungal diversity and reconstruct phylogenies, it is important to understand the potential effect that sequences with intragenic SPV can have on these studies.

By comparing the behavior of a contrived chimeric SSU rDNA sequence with that of a SSU rDNA sequence with suspected intragenic SPV, we evaluate the effects of such sequences on maximum parsimony analyses. The objective of this study is to examine the phylogenetic behavior of fungal SSU rDNA sequences with intragenic spatial phylogenetic variation. We test the efficacy of proposed chimera detection methods and report the effect sequences with intragenic SPV have on maximum parsimony analyses in light of taxon sampling sensitivities and differential taxon sampling.

4.3 Methods and Materials

4.3.1 Nucleotide Sequences

Nuclear ribosomal SSU and LSU DNA sequences were obtained from several sources (Table 4.1) or determined in previous studies by the authors (Platt and Spatafora, in review; Platt and Spatafora, in press,1999). The *Thamnolia subuliformis* nrSSU rDNA sequence was determined in another laboratory by several workers (Spatafora et al., unpublished). A chimeric nrSSU DNA sequence was contrived and synthesized using a cut and paste method in a multiple sequence alignment file. The chimera was spliced together at nucleotides 500-501, so that the first 500 bp from the 5' end were from *Cudonia circinans* and the remaining nucleotides (from 501 to the 3' end) were from *Leotia lubrica*..

Table 4.1 Sequences used in this study of SPV. SSU and LSU source numbers refer to GenBank Accession numbers.

Name	Species	SSU source	LSU source
Baeomyces (1)	*B. rufus*	AF113717	AF107558
Baeomyces (2)	*B. rufus*	AF113718	AF113743
Baeomyces 3)	*B. placophyllus*	AF113719	AF107560
Bryoglossum	*B. gracile*	-	-
Chimera	NA	Cudonia/Leotia(1)	Leotia (1)
Chlorociboria	*C. aeruginosa*	-	-
Cudonia	*C. circinans*	-	AF107553
Dibaeis	*D. baeomyces*	AF113712	AF113730
Discina	*D. macrospora*		U42678
Evernia	*E. prunastri*	AF113721	AF113745
Fabrella	*F. tsugae*		
Geoglossum	*G. glabrum*		AF113738
Gyromitra	*G. esculenta*	U42648	U42675
Heyderia	*H. abietis*		
Hypocrea	*H. lutea*		U00739
Icmadophila	*I. ericetorum*	AF113709	AF107556

Table 4.1 (Continued)

Leotia (1)	*L. lubrica*	L37536	
Leotia (2)	*L. viscosa*	AF113715	AF113737
Loramyces	*L. juncicola*		
Loxosporopsis	*L. corallifera*	AF113722	
Microglossum	*M. viride*		
Mitrula	*M. elegans*		
Morchella	*M. e?*		
Neobulgaria	*N.*		
Neurospora	*N. crassa*		
Ophiostoma	*O. piliferum*		
Sclerotinia	*S. sclerotiorum*		
Siphula (1)	*S. ceratites*	U72712	AF107557
Siphula (2)	*S. coriacea*		AF113724
Siphula (3)	*S. polyschides*	AF113710	AF113725
Spathularia	*S. velutipes*		AF113734
Stictis	*S. radiata*		AF113746
Thamnolia	*T. subuliformis*	****	AF113733
Trichoglossum	*T. hirsutum*		
Tuber	*T. gibbosum*	U42663	U42690
Vibrissea	*V. truncorum*		

4.3.2 Sequence Alignment and Variability

The biosequence editor SeqApp version 1.9a169 was used to create a single alignment file (Gilbert, 1992) . This master alignment file was comprised of all taxa (including the contrived chimera) and characters (SSU and LSU) utilized in this study. SWAN (Sliding Window Analysis) version 1.0 was used to analyze and graphically represent patterns of observed variability along the aligned nucleotide sequences of SSU rDNA only (Proutski and Holmes, 1997). For the SSU portion of the master alignment, variability distribution was estimated as an entropy function of the nucleotide variation observed using the following equation: $-\sum ni/N \ln ni/N$ ($i = a, c, g, t$). The window size was assigned by the program based on searches for the most informative window size.

This process records the number of variability values which fall outside the standard deviation about the mean for the whole alignment as the alignment is analyzed with various window sizes. The window size which resulted in the largest number of significantly deviating values was chosen as the most sensitive to nonrandom variation in the distribution of variability along the alignment (Proutski and Holmes, 1997).

4.3.3 Chimera Detection Software

CHIMERA_CHECK version 2.7 (Larsen et al., 1993) was used to detect spatial phylogenetic variation in the both the *Thamnolia* sequence and the contrived chimera. Three sequences presumed free of SPV, *Vibrissea* , *Dibaeis,* and *Spathularia* SSU were also analyzed. These "control" sequences were also run through the CHIMERA_CHECK search so that results could be more easily compared. All analyses were done on-line via access through the Ribosomal Database Project II web site (http://www.cme.msu. edu/RDP/analyses.html). The histogram results obtained from CHIMERA_CHECK were replotted onto a single graph for ease of comparison. The difference between the total number of shared oligomers of two specified fragments with similar server sequences and the total number of shared oligomers for the entire input sequence with the single most similar server sequence is plotted along the y-axis. The larger the difference the stronger the evidence that the input sequence is from two different organisms. This observed difference is measured every tenth position (x-axis) and based on comparisons of maps of 7-base oligomers.

4.3.4 Phylogenetic Analyses

The master alignment was used as the basis for all phylogenetic analyses. Selective taxon and character-removal were carried out using the "Delete – Restore Taxa" and "Include – Exclude Characters" commands in PAUP*4.0b2a (Swofford, 1999). Maximum parsimony analyses were carried out using PAUP*4.0b2a (Swofford, 1999). In all cases, gaps were treated as missing data with no additional weighting imposed. Unless specified otherwise, analyses utilized 1000 replicates of random sequence addition with a TBR branch-swapping algorithm and MULPARS in effect. 1000 bootstrap replicates were employed using the fast stepwise addition option and 1000 replicates of simple sequence addition. Adams consensus cladograms were computed for each analysis that yielded more than a single most parsimonious tree.

The partition homogeneity test (PHT), otherwise known as the incongruence length difference (ILD) test, was implemented in PAUP*4.0b2a (Swofford, 1999) on the character partitions of nrSSU and nrLSU using a heuristic search of ten replicates plus ten replicates of random sequence addition with TBR branch swapping. The partition homogeneity test (PHT) was also carried out on the 5' end versus 3' end character partitions of nrSSU only. The 5' versus 3' character partitions were delimited at the boundary between nucleotide 500 and 501; the break-point for the contrived chimera. These analyses utilized 100 replicates of simple sequence addition with the NNI branch swapping algorithm. Due to the low number of parsimony informative characters in such small character partitions and the large number of trees generated, a maximum of 1000 trees were saved. In all PHTs parsimony-uninformative characters were excluded from

the analyses so that only variable characters were used in calculating the statistics of PH partitions and randomized partitions (Cunningham, 1997) .

Each of the four basic taxon sampling schemes were also subjected to a permutation tail probability (PTP) test (Faith and Cranston, 1991) as implemented in PAUP*4.0b2a (Swofford, 1999). PTP tests utilized 10 replicates of a heuristic search with 10 replicates of random sequence addition and TBR branch swapping algorithm. In all PTP tests, only ingroup taxa were randomized. This rationale stems from the observation that the often strongly supported outgroup nodes can overshadow loss of resolution in less strongly supported ingroup nodes and thus give positively misleading results (Cunningham, 1997) .

4.4 Results

Multiple sequence alignment reveals that the 5' end of the *Thamnolia subuliformis* SSU rDNA sequence had higher nucleotide identity with *Dibaeis* and *Siphula* but the 3' end has higher nucleotide identity with *Cudonia* and *Spathularia* (Figure 4.1). If parsimony optimality criteria are employed these patterns may translate into 5' end synapomorphies with *Dibaeis* and *Siphula* but 3' end synapomorphies with *Cudonia* and *Spathularia*.

The sliding window analysis of SWAN version 1.0 did not reveal any significant spatial patterns of nucleotide variability that might be suggestive of chimeric sequences. However, the general pattern discerned from the variability distribution plot (Figure 4.2) confirms previous findings that the 3' end of nrSSU has higher variability (Eriksson,

1995). The mean variability distribution was calculated at 0.111328 with an 0.198835 standard deviation.

Figure 4.1 Multiple Sequence Alignment showing identities with different sets of taxa. Boxes reveal shifting signatures.

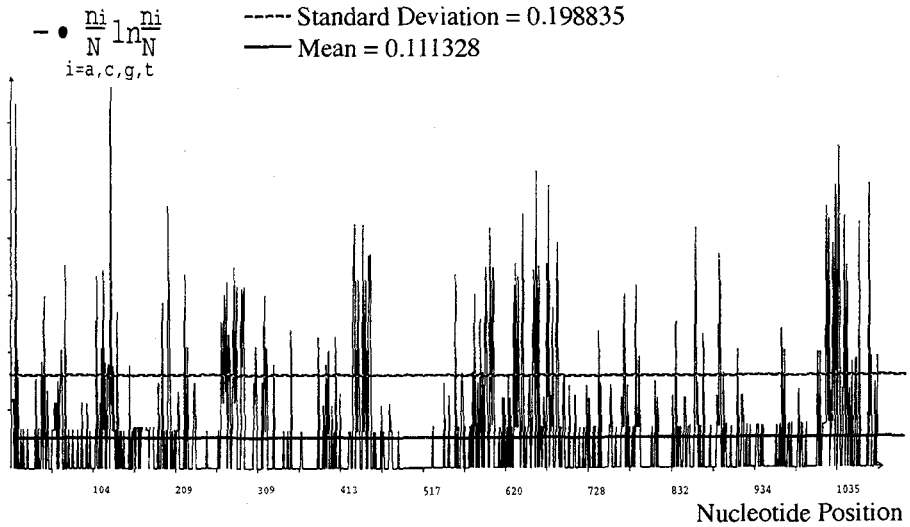
~265 bp downstream of 5' end of nuclear SSU rDNA amplicon

Cudonia circinans	GATGGTAGGATAGTGGCCTACCATGGTTTC
Spathularia flavida	GATGGTAGGATAGTGGCCTACCATGGTTTC
Thamnolia subuliformis	**GATTGTTGGGTAGTGGCCAACAATGGTGTT**
Dibaeis baeomyces	GATTGTTGGGTAGTGGCCAACAATGGTGTT
Siphula pickeringii	GATTGTTGGGTAGTGGCCAACAATGGTGTT

~425 bp downstream of 5' end of nuclear SSU rDNA amplicon

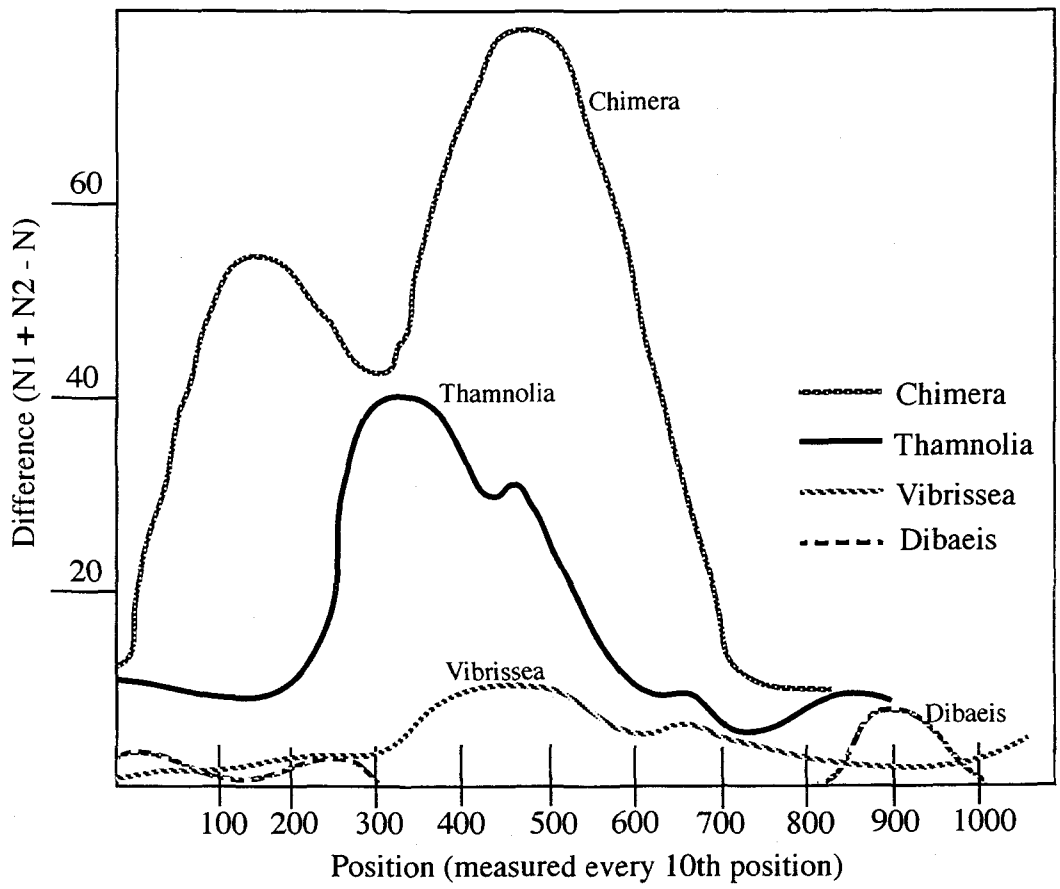
Cudonia circinans	GATACAGGGCTCTTTTGGGTCTTGTAATTG
Spathularia flavida	GATACAGGGCTCTTTTGGGTCTTGTAATTG
Thamnolia subuliformis	**GATACAGGGCCCTTTTGGGTCTTGTAATTG**
Dibaeis baeomyces	GATCTGGGGCATTTTTATGTCTCAGAATTG
Siphula pickeringii	GATCTGGGGGTCTTTAGGCTCTCAGAATTG

Figure 4.2 Estimated variability distribution as function of entropy.



Chimera check results were suggestive of the *Thamnolia* sequence being chimeric in origin (Figure 4.3). The contrived chimera sequence, which approximates a positive control in this study, also yielded results suggestive of a chimera. It is important to note that this software does not make a determination regarding the chimeric nature of the input sequence. However, a large difference of N1 + N2 versus N (total) at several 100bp fragment (oligomer) sites observed for *Thamnolia* and the contrived chimera is strongly suggestive of chimeric sequences (Figure 4.3). A roughly of bell-shaped curve is expected if a chimeric sequence is analyzed as an un-interrupted rise towards a single

Figure 4.3 Plotted curves of 4 sequences input into CHIMERA_CHECK.



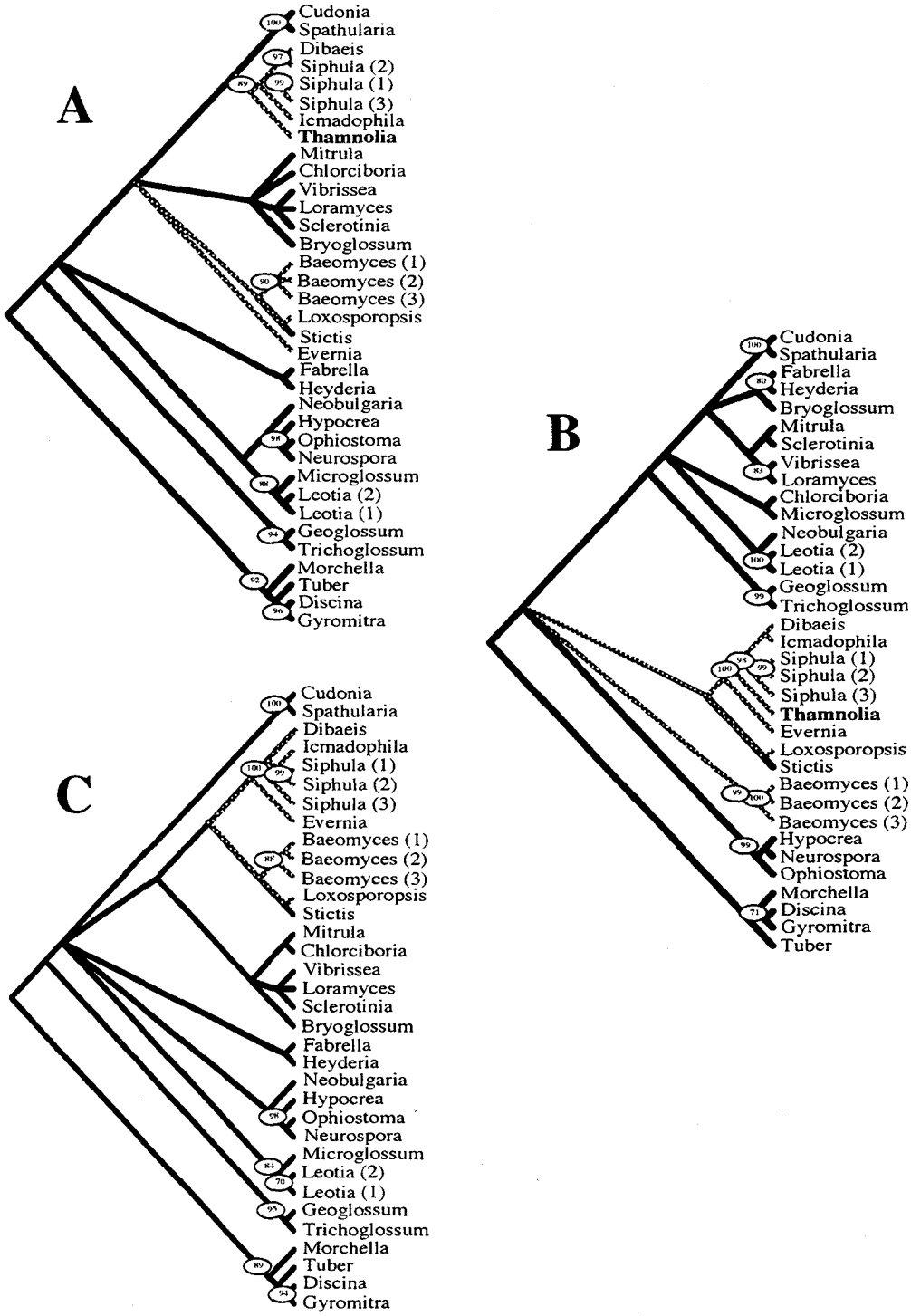
break-point from both sides is observed (Larsen et al., 1993). The three additional sequences assumed free of SPV that were also analyzed with CHIMERA_CHECK did not display the predicted chimera curve and exhibited a maximum difference of less than 15. This small difference is suggested to be too low to reveal chimerism (Larsen et al., 1993). *Spathularia* could not be plotted because it failed to be broken into two chimeric halves and thus the combined match of the halves with the database was better than that of the full length sequence (data not shown).

Maximum parsimony analysis of 206 parsimony-informative characters from the SSU portion of the master alignment without the contrived chimera resulted in a total of six equally most parsimonious trees representing three islands. The trees were 618 steps with a CI of 0.464 and RI of 0.602. The Adams consensus cladogram (Figure 4.4 – A) shows that the inclusion of *Thamnolia* places the Icmadophila clade sister to *Cudonia* and *Spathularia*. The inferred phylogeny also suggests a minimum of two independent gains of the lichen symbiosis for the taxa sampled (patterned branches).

Maximum parsimony analysis of 229 parsimony-informative characters from the LSU portion of the master alignment without the contrived chimera produced three equally most parsimonious trees of 1179 steps and a CI of 0.427 and RI of 0.580. The Adams consensus cladogram suggests a minimum of a single gain of the lichen symbiosis (patterned branches) although the critical node is unresolved (Figure 4.4 – B). The *Icmadophila* clade is placed sister to the lichenized taxon *Evernia* and is distant from *Cudonia* and *Spathularia*.

Maximum parsimony analysis of 198 parsimony-informative characters from the SSU portion of the master alignment without *Thamnolia* or the contrived chimera

Figure 4.4 Topological incongruency of SSU and LSU rDNA maximum parsimony inferred phylogenies. The Adams consensus cladogram for the SSU portion of the master data set including *Thamnolia* (A), the Adams consensus cladogram for the LSU portion of the master data set (B) and the Adams consensus cladogram for the SSU portion of the master data set excluding *Thamnolia* (C).



produced 13 equally most parsimonious trees representing a single island. The trees were 606 steps with a CI of 0.474 and an RI of 0.604. The Adams consensus cladogram shows that removing *Thamnolia* SSU from the data set yields a more similar topology with that inferred from LSU rDNA alone (Figure 4.4 – C). A single gain of the lichen symbiosis is inferred with the *Icmadophila* clade being placed as a sister taxon to the lichen *Evernia.*

In each of these taxon and character sampling schemes, the *Icmadophila* clade is well-supported with bootstrap values of 89 and 100 (Figure 4.4). However, no significant bootstrap support exists for the sister relationship of the *Icmadophila* clade with *Evernia* or *Cudonia* and *Spathularia*. Patterned branches reveal that fewer independent gains of the lichen symbiosis are inferred when the *Thamnolia* SSU sequence with intragenic SPV is removed from the analysis.

Selective taxon removal in maximum parsimony analyses of SSU rDNA reveal the topological impact of sequences with intragenic SPV on inferred phylogenies (Figure 4.5). Maximum parsimony analysis of 206 parsimony-informative characters of SSU rDNA including the *Thamnolia* sequence, result in six equally most parsimonious trees representing three islands (Table 4.2). This inferrence drawn from the Adams consensus cladogram, places the *Icmadophila* clade as a sister group to *Cudonia* and *Spathularia* and infers at least two independent gains of lichenization (shown with patterned branches) (Figure 4.5 – A). Removing the *Thamnolia* sequence from the data set (taxon-removal of *Thamnolia)* infers a single gain of lichenization (patterned branches) (Figure 4.5 – B). This Adams consensus cladogram of 13 equally most parsimonious trees of 606 steps representing a single island places the *Icmadophila* clade as a sister group to *Evernia* and more closely related to other lichenized taxa (i.e., the *Baeomyces* clade).

Taxon removal of the contrived chimera reveals similar patterns of topological incongruence. Maximum parsimony analysis of 199 parsimony-informative characters of SSU rDNA with the contrived chimera (but without *Thamnolia*) results in 17 equally most parsimonious trees of 611 steps representing two islands (Table 4.2). The Adams consensus cladogram (Figure 4.5 – C) shows that including the contrived chimera infers a phylogeny where *Microglossum* and *Leotia* are a sister group to the clade comprised of *Spathularia, Fabrella,* and *Heyderia*. However, no significant bootstrap support exists for this relationship. Maximum parsimony analysis with taxon-removal of the contrived chimera produced 13 equally most parsimonious trees of 602 steps representing a single island (Table 4.2). The Adams consensus cladogram (Figure 4.5 – D) reveals that removing the contrived chimera infers a more disparate relationship for *Microglossum* and *Leotia* with either *Spathularia* or *Fabrella* and *Heyderia*. Furthermore, *Spathularia* no longer appears as a sister taxon to *Fabrella* and *Heyderia*. This inference is consistent with other SSU and LSU rDNA-based phylogenetic studies which place *Spathularia* as a sister taxon to members of the Rhytismatales, rather than inferring a close relationship with members of the Helotiales, such as *Leotia, Microglossum, Fabrella,* and *Heyderia* (Gernandt, 1998; Platt and Spatafora, 1998).

Differential character sampling of the 5' and 3' ends of SSU rDNA in conjunction with selective taxon removal was also carried out with maximum parsimony analyses. The 5' end consisted of the first 500 bp of SSU rDNA while the 3' end included all of the remaining SSU rDNA characters. The inferred phylogenies (data not shown) revealed very little topological congruence, most likely as an artifact of the paucity of parsimony

Figure 4.5 Selective taxon removal approach. Maximum parsimony analyses of SSU rDNA showing the effects of inferred phylogenies with and without sequences with intragenic spatial phylogenetic variation (SPV). Adams consensus cladograms under various taxon-removal schemes are shown. A) includes the *Thamnolia* sequence, B) taxon-removal of *Thamnolia*, C) includes the contrived chimera, and D) taxon-removal of the contrived chimera. Comparing cladograms A and C approximates a positive control, while comparing cladograms B and D approximates a negative control.

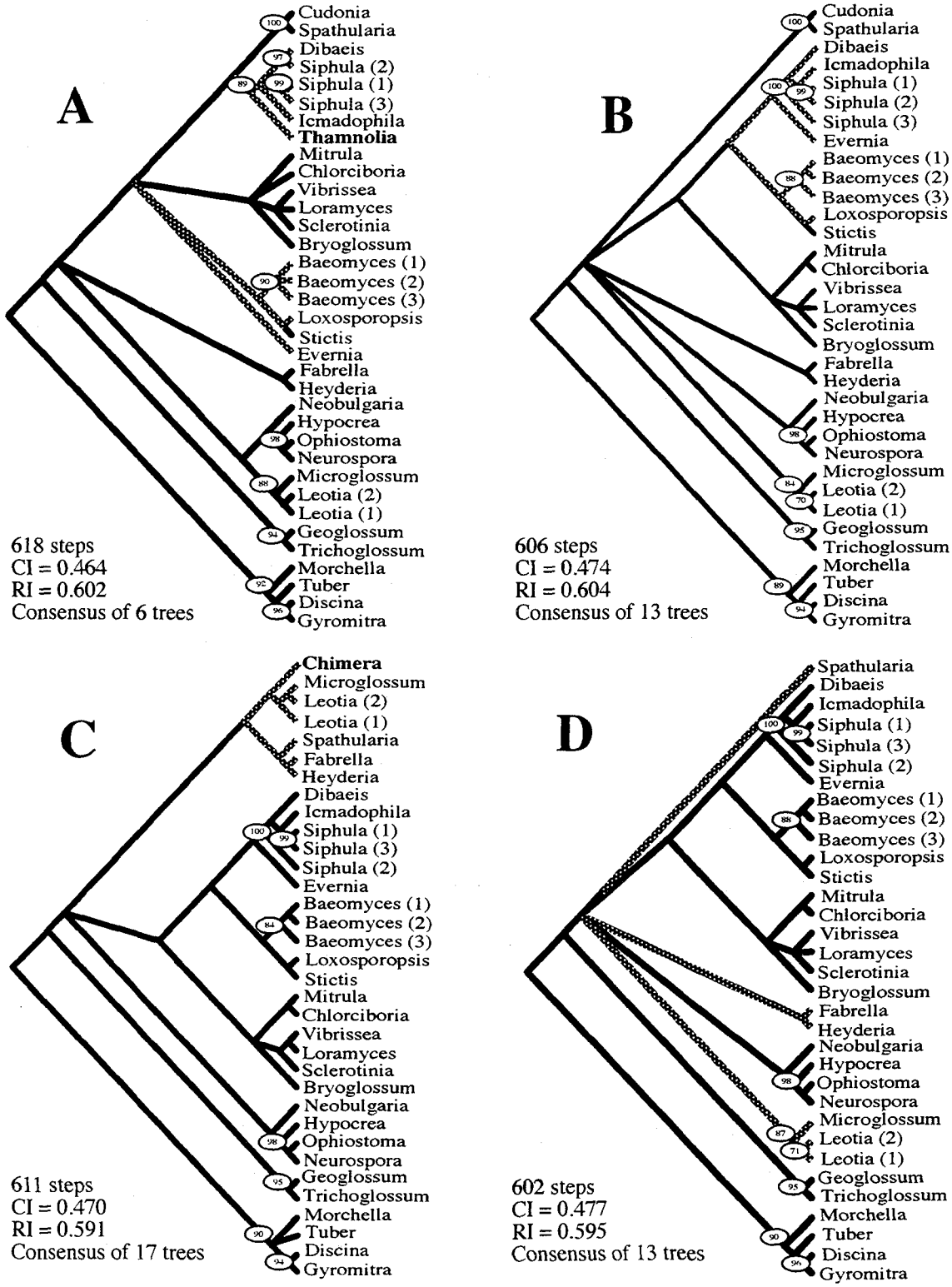

A
Cudonia
Spathularia
Dibaeis
Siphula (2)
Siphula (1)
Siphula (3)
Icmadophila
Thamnolia
Mitrula
Chlorciboria
Vibrissea
Loramyces
Sclerotinia
Bryoglossum
Baeomyces (1)
Baeomyces (2)
Baeomyces (3)
Loxosporopsis
Stictis
Evernia
Fabrella
Heyderia
Neobulgaria
Hypocrea
Ophiostoma
Neurospora
Microglossum
Leotia (2)
Leotia (1)
Geoglossum
Trichoglossum
Morchella
Tuber
Discina
Gyromitra
618 steps
CI = 0.464
RI = 0.602
Consensus of 6 trees
B
Cudonia
Spathularia
Dibaeis
Icmadophila
Siphula (1)
Siphula (2)
Siphula (3)
Evernia
Baeomyces (1)
Baeomyces (2)
Baeomyces (3)
Loxosporopsis
Stictis
Mitrula
Chlorciboria
Vibrissea
Loramyces
Sclerotinia
Bryoglossum
Fabrella
Heyderia
Neobulgaria
Hypocrea
Ophiostoma
Neurospora
Microglossum
Leotia (2)
Leotia (1)
Geoglossum
Trichoglossum
Morchella
Tuber
Discina
Gyromitra
606 steps
CI = 0.474
RI = 0.604
Consensus of 13 trees
C
Chimera
Microglossum
Leotia (2)
Leotia (1)
Spathularia
Fabrella
Heyderia
Dibaeis
Icmadophila
Siphula (1)
Siphula (3)
Siphula (2)
Evernia
Baeomyces (1)
Baeomyces (2)
Baeomyces (3)
Loxosporopsis
Stictis
Mitrula
Chlorciboria
Vibrissea
Loramyces
Sclerotinia
Bryoglossum
Neobulgaria
Hypocrea
Ophiostoma
Neurospora
Geoglossum
Trichoglossum
Morchella
Tuber
Discina
Gyromitra
611 steps
CI = 0.470
RI = 0.591
Consensus of 17 trees
D
Spathularia
Dibaeis
Icmadophila
Siphula (1)
Siphula (3)
Siphula (2)
Evernia
Baeomyces (1)
Baeomyces (2)
Baeomyces (3)
Loxosporopsis
Stictis
Mitrula
Chlorciboria
Vibrissea
Loramyces
Sclerotinia
Bryoglossum
Fabrella
Heyderia
Neobulgaria
Hypocrea
Ophiostoma
Neurospora
Microglossum
Leotia (2)
Leotia (1)
Geoglossum
Trichoglossum
Morchella
Tuber
Discina
Gyromitra
602 steps
CI = 0.477
RI = 0.595
Consensus of 13 trees

Figure 4.5

informative characters (Table 4.3). The degree of resolution also varied greatly with 25 to 1000 equally most parsimonious trees being generated in each heuristic search (max trees set at 1000) (Table 4.3). Under all taxon sampling schemes, the CI calculated from the 3' end analysis was higher than that of the 5' end and likewise, the RI calculated from the 3' end analysis was higher than that of the 5' end. This is most likely a reflection of the larger number of parsimony informative characters present in the 3' end.

Selective taxon-removal was also performed and evaluated under the partition homogeneity and PTP tests (Table 4.3). When *Thamnolia* is included, the SSU versus LSU partitions are not significantly incongruent (P = 0.4300) however, congruence is marginally improved when *Thamnolia* is excluded (P = 0.500) (Table 4.4). The SSU versus LSU data partitions are not highly congruent when the Chimera is included (P = 0.1700) but congruence does increase when the Chimera is excluded (P = 0.4400) (Table 4.4). Only the SSU 5' versus 3' data partitions show significant incongruence (P = 0.0100). Under all taxon sampling schemes, the PTP test of the SSU partition yielded a P value of 0.0100 suggesting that the null hypothesis that the data are randomly structured must be rejected. This result indicates that significant phylogenetic signal exists in the data set under all of the taxon-removal conditions.

Table 4.2 Maximum parsimony scores for taxon-removal analyses of the SSU rDNA. A heuristic search with 1000 replicates of random sequence addition and TBR branch-swapping was employed.

Taxon Sampling	Informative characters	Score	Islands	# of trees	CI	RI
+ *Thamnolia*	206	618	3	6	0.464	0.602
- *Thamnolia*	198	606	1	13	0.474	0.604
+ Chimera	199	611	2	17	0.470	0.591
- Chimera	198	602	1	13	0.477	0.595

Table 4.3 Maximum parsimony scores for phylogenies of the 5' versus 3'ends under different taxon-removal schemes. Maximum parsimony analyses utilized 1000 replications of random sequence addition with a TBR branch-swapping algorithm and max trees set to 1000.

Taxon Sampling	Partition	Informative characters	Islands	# of Trees	Score	CI	RI
+*Thamnolia*	5' end	96	1	1000	401	0.628	0.622
+*Thamnolia*	3' end	110	1	36	421	0.639	0.652
-*Thamnolia*	5' end	94	3	270	395	0.638	0.619
-*Thamnolia*	3' end	104	1	104	417	0.640	0.651
+ Chimera	5' end	94	1	256	395	0.638	0.619
+ Chimera	3' end	105	1	92	415	0.643	0.648
- Chimera	5' end	93	1	25	392	0.640	0.617
- Chimera	3' end	105	1	92	415	0.643	0.639

Table 4.4 Tree scores and test results for the 4 subsets of data analyzed.

Taxon Sampling	PHT SSU vs. LSU	PHT SSU 5' vs. 3'	PTP test SSU
+ *Thamnolia*	P = 0.4300	0.0100	P = 0.0100
- *Thamnolia*	P = 0.5000	0.0100	P = 0.0100
+ Chimera	P = 0.1700	0.0100	P = 0.0100
- Chimera	P = 0.4400	0.0100	P = 0.0100

4.5 Discussion

The multiple sequence alignment (Figure 4.1) reveals that *Thamnolia* has potential synapomorphies with *Dibaeis* and *Siphula* around 265 bp from the start of the SSU rRNA region. These synapomorphies represent two transitions and five transversions with respect to the other taxa in the multiple sequence alignment (i.e., *Cudonia* and *Spathularia*). At about 425 bp downstream of the 5' end of SSU rDNA,

Thamnolia has apparent synapomorphies with *Cudonia* and *Spathularia*. These synapomorphies represent four transitions and two transversions with respect to the other taxa in the multiple sequence alignment (i.e., *Dibaeis* and *Siphula*). These results suggest that if recombination (PCR-mediated or natural) is the cause of the intragenic SPV in this sequence, the event occurred in the conserved region around 300 – 400 bp downstream of the 5' end of the SSU rRNA gene. And while it is not outside of the realm of possibility that SSU rDNA sequences may actually evolve in a manner which would result in this pattern, this multiple sequence alignment scheme is suggestive of a chimeric sequence (Wang and Wang, 1997) . Because this *Thamnolia* SSU rDNA sequence was determined as part of another study (Spatafora et al., unpublished), we do not have the necessary information to further pursue an in-depth investigation of the source of this intragenic SPV (e.g., recombination, PCR-mediated chimera formation, or chimerazation resulting from contig assembly of non-conspecific sequences). For the purposes of this study, we focus on the behavior of intragenic SPV in maximum parsimony analyses.

The plot of variability distribution as a function of entropy estimated with the sliding window analysis of SWAN version 1.0 did not reveal any obvious extremes that would be suggestive of a chimeric sequence (Figure 4.2). However, it should be noted that if intragenic SPV arises from a chimera which is comprised of two closely related taxa or both taxa are represented in the alignment, SWAN would likely fail to detect abrupt variability distribution.

CHIMERA_CHECK was effective in detecting intragenic SPV and produced results suggestive that *Thamnolia* and the contrived chimera were composed of two chimeric halves (Figure 4.3). For the *Thamnolia* sequence, a large difference of N1+N2

– N (40) was calculated around the nucleotide positions where recombination may have occurred if this intragenic SPV is due to this phenomenon. Furthermore, an even larger difference of N1+N2 – N = 80 was calculated at the nucleotide positions where the contrived chimera was spliced. This sharp bell-shaped curve with a large difference is highly suggestive of a chimeric sequence (Larsen et al., 1993). It should be noted however, that the contrived chimera was composed of one sequence which was from GenBank and another sequence which has a very close relative in GenBank (i.e., another species of the same genus). Therefore, it is expected that the contrived chimera would produce such a sharp curve and large difference since the two halves would have such close matches (or "hits) for different GenBank sequences. Because CHIMERA_CHECK is database dependent it would likely not detect a chimera between two closely related taxa, especially if those taxa were not represented in GenBank (Wang and Wang, 1997). Furthermore, the danger also exists that some of the sequences deposited in GenBank are themselves chimeric and this could be problematic if the sequence being tested is a "hit" with a chimeric sequence. It is reassuring however, that even though CHIMERA_CHECK could result in a false negative (indicating that no chimera exists), it did not appear to give any false positive results on the sequences tested. For *Dibaeis* and *Vibrissea* which have no close relatives represented in GenBank, the curve did not reflect a large difference of N1+N2 – N. Similarity score differences also did not support potential chimeras as the highest similarity score for the full length sequence was not lower than those of both fragments (Larsen et al., 1993). In addition, *Spathularia*, which does have a close relative represented in GenBank (i.e., a different species of *Spathularia*), failed to be split into two chimeric halves.

Adams consensus cladograms from maximum parsimony analyses with selective taxon and character removal reveal that greater topological congruence results with respect to SSU and LSU portions of rDNA when *Thamnolia* is excluded from the SSU data set (Figure 4.4). Including *Thamnolia* in maximum parsimony analyses of the SSU data set, infers a phylogeny in which lichenization has arisen at least two times independently for the taxa sampled (Figure 4.4 – A). *Thamnolia* forms a well-supported clade with other members of the Icmadophilaceae (*Icmadophila, Dibaeis,* and *Siphula*). This phylogeny was better resolved than the phylogeny which excludes *Thamnolia* (Figure 4.4 – C), in having six equally most parsimonious trees, rather than 13. However, these six trees represent three islands, and the phylogeny excluding *Thamnolia* resulted in a single island (Table 4.2). This loss of resolution may be explained by the reduction in parsimony-informative characters which goes from 206 when *Thamnolia* is included to 198 when it is excluded (Table 4.2). A difference in consistency index (CI) is also observed. Maximum parsimony trees from SSU data including *Thamnolia* give a CI of 0.464 while excluding *Thamnolia* slightly reduces the amount of homoplasy to CI = 0.474 (Table 4.2). The increased homoplasy observed when *Thamnolia* is included is probably not entirely a result of intragenic SPV, but is most likely a simple reflection of the number of included taxa. Although trees B and C (Figure 4.4) do not have identical topologies, single gains of lichenization are inferred from LSU nrDNA and SSU when *Thamnolia* is excluded. The Icmadophilaceae clade remains well supported but bootstrap support for this clade with other lichenized taxa is not substantial.

The contrived chimera was utilized to approximate a positive control for comparison of behavior in maximum parsimony analyses. Taxon-removal of both the

Thamnolia sequence and the contrived chimera reveal that vastly different phylogenies are inferred when sequences with intragenic SPV are included in the sampling (Figure 4.5). As previously addressed, including the intragenic SPV sequence of *Thamnolia* in SSU rDNA maximum parsimony analysis, infers at least two independent gains of lichenization (Figures 4.4 – A & 4.5 – A). Removing this sequence infers a single gain of lichenization under maximum parsimony criteria (Figure 4.5 – B). Selective taxon-removal of the contrived chimera exhibited similar behavior in maximum parsimony analyses. When the chimera is included, it forms a clade with *Microglossum* and *Leotia* ; this clade is a sister group to a clade comprised of *Spathularia, Fabrella*, and *Heyderia* (Figure 4.5 – C). Thus, including the chimera results in a single superclade containing the chimera, *Microglossum, Leotia, Spathularia, Fabrella,* and *Heyderia*. (patterned branches in Figure 4.5 – C). However, when the chimera is excluded, this superclade becomes three disparate assemblages; a relatively basal *Microglossum* and *Leotia* clade, an intermediate *Fabrella* and *Heyderia* clade, and *Spathularia* in a derived position (patterned branches in Figure 4.5 – D). It is important to note that no bootstrap support exists for the superclade that appears in the phylogeny inferred from maximum parsimony analysis including the chimera. Furthermore, no support exists within the clade. However, when the chimera is excluded, the *Microglossum* and *Leotia* clade has good bootstrap support.

Selective taxon-removal of the contrived chimera also improves resolution and the level of observed homoplasy (CI)(Table 4.2). However, as in the taxon removal of *Thamnolia*, the CI may be more reflective of the number of included taxa, than properties of intragenic SPV. The improved resolution of taxon-removal of the chimera compared

with the *Thamnolia* taxon-removal may be explained by the number of parsimony informative characters. Although removing *Thamnolia* from the taxon sampling resulted in the loss of eight parsimony informative characters, removing the contrived chimera only resulted in the loss of a single parsimony informative character (Table 4.2). This retention of parsimony-informative characters is the most reasonable explanation for the improved resolution in the chimera example.

Comparing the maximum parsimony Adams consensus cladograms B and D in Figure 4.5 approximates a negative control and is illustrative that the observed topologies are not simply the result of differential taxon sampling, but taxon removal specifically of sequences with intragenic SPV. Cladogram B which includes *Cudonia* has an identical topology to that of Cladogram D from which *Cudonia* is excluded. In addition, bootstrap support is nearly identical at all nodes in both topologies (Figure 4.5 – B & D). Both analyses yield equal resolution of 13 trees based on 198 parsimony informative characters, even though the number of steps differs by four (Table 4.2). The amount of observed homoplasy is once again reduced under selective taxon-removal as excluding *Cudonia* increases the CI from 0.474 to 0.477.

Results from the PTP tests suggest that the data are not randomly structured (P = 0.0100 PTP, rejecting null hypothesis) and therefore have phylogenetic signal. Although this test has been criticized for several reasons (Alroy, 1994; Bryant, 1992; Carpenter, 1992; Farris et al., 1994), it was utilized here simply as a comparative measure to determine if changes in taxon sampling caused any significant losses of phylogenetic signal. These results demonstrate that the selective taxon-removal in this study does not result in a measurable loss of phylogenetic signal (Table 4.4). Therefore, any topological

changes observed in maximum parsimony analyses subjected to selective taxon-removal are more likely to be due to the intragenic SPV present in the nucleotide sequences themselves rather than a loss of phylogenetic signal. This conclusion was also demonstrated with comparing cladograms B and D in Figure 4.5. The PTP test has been shown to be of limited value in discriminating data that will produce trees with significant confidence and resolution (Slowinski and Crother, 1998). However, we have not used the PTP test here as a measure of confidence and our intent is not to infer an accurate or confident, highly resolved phylogeny, but simply to make observations regarding the behavior of sequences with intragenic SPV in maximum parsimony methods.

Selective taxon-removal with the partition homogeneity test of the 5' versus 3' ends of SSU rDNA failed to show congruence ($P = 0.0100$) (Table 4.4). Incongruence would be predicted for taxon sampling that includes sequences with intragenic SPV, or specifically for chimeric sequences, if partitions are designated at the break-point of the chimera. However, even when sequences with intragenic SPV were excluded, significant incongruence was retained. This may again, be an artifact of the paucity of parsimony-informative characters present in these smaller fragments of SSU rDNA.

The PHT results from the SSU and LSU partitions under selective taxon-removal did not show any significant incongruence even when the contrived chimera was included (Table 4.4). Congruence did however, improve when the sequences with intragenic SPV were excluded from the analyses. The improvement is especially notable in the case of the contrived chimera when the PH test p-value increased from 0.1700 to 0.4400 (Table 4.4). This inability to detect incongruence indicates the partition homogeneity test is not

an effective means for screening potential chimeras or sequences with intragenic SPV, at least with nuclear SSU rDNA data. Furthermore, it confirms Cunningham's observation that congruence between data partitions does not necessarily imply phylogenetic accuracy (Cunningham, 1997). The partition homogeneity test showed increased congruence when *Thamnolia* was excluded (Table 4.4) however, the resolution also decreased from six trees to 13 trees (Table 4.2). Cunningham noted that congruence can be a result of poorly resolved data (Cunningham, 1997) and this should be considered a factor here since SSU rDNA has been shown to have poor resolving power in broad-scale ascomycete phylogenies (Berbee, 1996; Spatafora, 1995).

Selective taxon-removal in maximum parsimony analyses has revealed the impact that SSU rDNA sequences with intragenic SPV have on inferred phylogenies. In some cases, taxon-removal may even be an effective way of screening for such sequences. However, caution should be used as phylogenetic analyses are sensitive to taxon and character sampling and artifacts of such data may also affect the subsequent topologies. In the examples we present, taxon sampling did not appear to be an issue as taxon-removal of a non-SPV sequence did not affect topology (Figure 4.5 – B & D). The failure of some methods, (e.g., PHT, SWAN, and potentially others) to predict intragenic SPV should serve as caution to anyone relying on these methods alone for screening sequences to be used in phylogenetic studies. No matter what methods are ultimately employed to infer phylogenies, accuracy can only be achieved with the use of good quality raw data.

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CHAPTER 5

Phylogenetic Reconstruction of the Earth Tongues (Geoglossaceae, Helotiales) Inferred from Nuclear Ribosomal DNA

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5.1 Abstract

This study utilizes phylogenetic analyses of nuclear SSU (small subunit) and LSU (large subunit) ribosomal DNA (rDNA) to construct phylogenetic hypotheses for the Earth Tongue family, Geoglossaceae (Helotiales, Ascomycota). The phylogenies presented were inferred from cladistic analysis of combined SSU and LSU rDNA nucleotide sequence data from exemplar taxa. A more inclusive taxon sampling of the Geoglossaceae is presented in LSU rDNA inferred phylogenies. Maximum likelihood methods were used to provide statistical evidence that the family Geoglossaceae as currently circumscribed, is not monophyletic. Fungi historically treated under this family concept are found in five unique lineages of the Ascomycota. Based on these data, a revision of the Geoglossaceae is suggested to include the genera *Geoglossum, Trichoglossum*, and *Sarcoleotia* which all possess brown-walled paraphyses. *Microglossum* is distinct from *Geoglossum* and *Trichoglossum* and shows greater affinity for *Leotia*. Cladistic analyses also show that *Cudonia* and *Spathularia* are more closely related to members of the Rhytismatales than to other members of the Geoglossaceae.

5.2 Introduction

The inoperculate discomycete family Geoglossaceae is comprised of some of the largest, and most distinctive fungi of the Helotiales. The fruitbodies (i.e., ascomata) are typically fleshy and clavulate, spathulate or stipitate. Several species are brightly colored orange or green, while many are brown or black. Despite the fact that these are among the largest and most conspicuous inoperculate discomycetes, the Geoglossaceae have

been overlooked with regards to cladistic studies, even despite advances in modern systematic technologies. Korf (1990) poignantly noted that, "few discomycete groups have been subjected to rigorous cladistic, or even phenetic, analysis and even fewer have been the subject of the newer molecular techniques that argur well to unravel many current mysteries of relationships." Certainly, the Geoglossaceae (and Helotiales, for that matter) fall within this category of mysterious and neglected groups of discomycetes.

The "mysteries of relationships" centered around the Geoglossaceae is evidenced by historically tenuous classifications. These unstable concepts central to the earth tongues, stem from broadly overlapping, likely convergent morphological characters. These characters include highly variable ascus and ascospore morphology. The ascospores are forcibly discharged from the asci through a pore or slit (i.e., inoperculate asci). The pore at the ascus apex may or may not blue when treated with iodine (e.g., Meltzer's reagent). The ascospores may be pigmented or hyaline, and range from aseptate to highly septate, fairly short elliptical to cylindrical, fusiform, or even clavate, depending on which familial classification is being recognized. The paraphyses are also quite variable, ranging from filiform, branched, aseptate to septate with hyaline or brown cell walls. The use of such characters has resulted in many conflicting classification hypotheses which do not necessarily reflect the phylogeny of earth tongues.

The first classification of the family was proposed by Boudier in 1885. Since then, many revised classifications have been proposed, most of these based on comparative morphology of ascus and ascospore characters (Saccardo, 1889; Schroeter, 1893; Massee, 1895, 1897; Durand, 1908; Clements and Shear, 1931; Imai, 1941, 1955; Nannfeldt, 1942; Mains, 1954, 1955; Maas Geesteranus, 1964; and others). This

relatively large body of literature demonstrates the fair amount of taxonomic attention paid to the Geoglossaceae. Despite such taxonomic focus, however, systematic and cladistic studies of the family are lacking. Perhaps this neglect is one reason that the generic limits within the Geoglossaceae are so poorly defined and held with such little confidence (Korf, 1990). Although the precise delimitation of the family is controversial, several core genera are generally agreed upon; these are *Geoglossum, Microglossum*, and *Trichoglossum*. Many other genera are highly contentious with respect to their relationship to the Geoglossaceae. Some of these genera have traversed the "nebulous borderland between the Geoglossaceae and Helotiaceae" (Maas Geesteranus, 1966). A good example of this dynamic is *Leotia lubrica* which has been classified in the Geoglossaceae (Dennis, 1977), Helotiaceae (Korf, 1958) and Leotiaceae (Hawksworth et al., 1996). This example is further confused by classification problems at the ordinal level. Chadefaud recognized such a disparate relationship between the family Helotiaceae and the genus *Leotia* that he suggested accommodation in two distinct orders, Helotiales and Leotiales (Chadefaud, 1960). These ambiguous and unstable hypotheses frustrated taxonomists over thirty years ago. Maas Geesteranus (1966) insightfully stated that "One problem remains: that of finding suitable characters that can be used to break up the unwieldy Helotiaceae." With the vast instability of delimitation between the Geoglossaceae and Helotiaceae (Leotiaceae), this statement may be generally applied to the earth tongues.

Current molecular technology now allows us to access those "suitable characters". The heritable characters of rDNA, so easily accessible now through PCR and nucleotide sequencing are ideal for helping us unravel the relationships within and among the

inoperculate discomycetes. These characters are useful contributions toward transcending the confusion presented by characters subject to convergent evolution, and may point to phylogenetically meaningful morphological characters. This study utilizes the characters of nuclear SSU and LSU rDNA to reconstruct the phylogenetic history of the earth tongues.

5.3 Materials and Methods

5.3.1 Taxon Sampling

Freshly collected fruitbodies or herbarium material (up to 36 years old) was used for extracting total genomic DNA. A portion of each fresh collection was dried and deposited as voucher material in the Oregon State University (OSC) Mycological Collection. Collection numbers for a given specimen can be obtained from the nucleotide query of GenBank. The taxa sampled are listed in Table 5.1.

5.3.2 Character Sampling

Total genomic DNA was extracted using one of two methods. Either a modified CTAB method with subsequent further DNA purification (Platt and Spatafora, In press) or a detergent method adapted for heavily pigmented fungi (Winton, pers. com). In this latter protocol, specimens were ground in liquid nitrogen or pulverized with a Mini-BeadBeater™ Type BX4 cell disrupter (BIOSPEC Products, Inc., Bartleville, OK). After a 40 minute 65°C incubation in lysis buffer (CTAB or SDS) with 1%

Table 5.1 Specimens included in this study and their ordinal and familial placements under the classification of Hawksworth et. al (1995). Specimens for which no GenBank accession number is given ("-") have been determined in this study and are not yet available on GenBank.

Species	Order	Family	SSU GenBank Accession No.	LSU GenBank Accession No.
Ascocoryne sarcoides	Helotiales	Leotiaceae	-	-
Bisporella citrina	Helotiales	Leotiaceae	NA	-
Bryoglossum gracile	Helotiales	Leotiaceae	-	-
Bulgaria inquinans	Helotiales	Leotiaceae	AJ224362	-
Chlorociboria aeruginosa	Helotiales	Leotiaceae	-	-
Colpoma quercinum	Rhytismatales	Rhytismataceae	-	-
Cudonia circinans	Helotiales	Geoglossaceae	AF107343	AF107553
Cudoniella clavus	Helotiales	Leotiaceae	NA	-
Discina macrospora	Pezizales	Helvellaceae	U42651	U42678
Fabrella tsugae	Helotiales	Hemiphacidaceae	-	-
Geoglossum arenarium	Helotiales	Geoglossaceae	NA	-
Geoglossum glabrum	Helotiales	Geoglossaceae	-	AF113738
Geoglossum nigritum	Helotiales	Geoglossaceae	NA	-
Geoglossum umbratile	Helotiales	Geoglossaceae	NA	-
Gyromitra esculenta	Pezizales	Helvellaceae	U42648	U42675
Heyderia abietis	Helotiales	Leotiaceae	-	-
Hypocrea lutea	Hypocreales	Hypocreaceae	D14407	U00739
Lachnum cf. tenuissimus	Helotiales	Hyaloscyphaceae	NA	-
Leotia lubrica	Helotiales	Leotiaceae	L37536	-
Leotia viscosa	Helotiales	Leotiaceae	AF113715	AF113737
Loramyces juncicola	Helotiales	Loramycetaceae	-	-
Microglossum atropurpureum	Helotiales	Geoglossaceae	NA	-
Microglossum fuscorubrens	Helotiales	Geoglossaceae	NA	-
Microglossum olivaceum	Helotiales	Geoglossaceae	NA	-
Microglossum rufum	Helotiales	Geoglossaceae	NA	-
Microglossum viride	Helotiales	Geoglossaceae	-	-
Mitrula elegans	Helotiales	Sclerotiniaceae	-	-
Mitrula paludosa	Helotiales	Sclerotiniaceae	NA	-
Morchella esculenta	Pezizales	Morchellaceae	U42642	U42669
Neobulgaria pura	Helotiales	Leotiaceae	U45444	-
Neolecta vittelina	Neolectales	Neolectaceae	Z27408	Z48318
Neurospora crassa	Sordariales	Sordariaceae	X04971	U40124
Ophiostoma piliferum	Ophiostomatales	Ophiostomataceae	U20377	U47837
Orbilia auricolor	Helotiales	Orbiliaceae	U72598	-
Orbilia delicatula	Helotiales	Orbiliaceae	U72603	-
Pezicula cinnamonea	Helotiales	Dermateaceae	-	-
Pezoloma kathieae	Helotiales	Leotiaceae	-	-
Sarcoleotia globosa	Helotiales	Leotiaceae	NA	-
Sclerotinia sclerotiorum	Helotiales	Sclerotiniaceae	L37541	Z73762
Sclerotinia veratri	Helotiales	Sclerotiniaceae	NA	AF113739
Spathularia velutipes	Helotiales	Geoglossaceae	AF107344	AF107554
Trichoglossum farlowii	Helotiales	Geoglossaceae	NA	-
Trichoglossum hirsutum	Helotiales	Geoglossaceae	-	-
Trichoglossum walteri	Helotiales	Geoglossaceae	NA	-
Tuber gibbosum	Pezizales	Tuberaceae	U42663	U42690
Vibrissea truncorum	Helotiales	Vibrisseaceae	-	-

Polyvinylpolypyrrolidone (PVPP), crude lysates were extracted once with Chloroform:Isoamyl alcohol (24:1). These extracts were then subjected to a slightly modified QIAamp® tissue protocol (QIAGEN® Inc., Valencia, CA). An equal volume of QIAamp® Buffer AL was added to the DNA extract, mixed by gentle vortexing, and incubated for 10 minutes at 70°C. One-half total volume of 100% ethanol was added and mixed by inversion prior to placing the sample on the QIAamp® spin column and completing the QIAamp® tissue protocol.

Polymerase chain reaction (PCR) amplification (Mullis and Faloona, 1987) of nuclear SSU and LSU rDNA was carried out using methods previously described (Platt and Spatafora, in press). PCR products were purified by precipitation (Platt and Spatafora, in press) prior to direct sequencing at the Center for Gene Research and Biotechnology's Central Service Laboratory at Oregon State University. Both the coding and template strands of PCR products were sequenced utilizing the primers NS2, NS4, NS5 (White et al., 1990), SR7R, SR11R (Spatafora et al., 1995), and NS24 (Gargas and Taylor, 1992) for SSU nrDNA and LR0R, LR3 (Vilgalys and Sun, 1994) for LSU nrDNA.

5.3.3 Sequence Analysis

SSU rDNA sequences were edited and aligned manually using the biosequence editor SeqApp version 1.9a169 (Gilbert, 1992). SSU rDNA introns were excluded from the combined data set. LSU rDNA sequences were edited in EditView 1.0.1 (The Perkin-Elmer Corp., 1996) and subjected to an initial alignment in ClustalX (Thompson et al., 1997). This initial alignment was then adjusted manually by visual, color-based estimation in SeqApp (Gilbert, 1992).

All phylogenetic analyses were performed in PAUP*4.0b2a (Swofford, 1999). The ambiguous regions (e.g., divergent domains) of the LSU rDNA were excluded from analyses of both the combined data set and the LSU data set. All ambiguously alignable gaps (single insertions) were treated as missing data, but nonambiguously alignable gaps were treated either as missing or as a fifth character state. The partition homogeneity test (or ILD test) (Mickevich and Farris, 1981; Farris et al., 1994) was performed in PAUP* to test for combinability of nuclear SSU and LSU rDNA data sets. Maximum parsimony analyses utilized heuristic searches and 1000 replicates of random sequence addition with the tree-bisection reconnection (TBR) branch swapping algorithm. The choice of *Neolecta* as the outgroup is based on the rationale that it is more desirable to choose a single closely related sister group than a nested succession of taxa (Smith 1994). Previous studies have shown that the Saccharomycetes are a sister group to the euascomycetes (Berbee and Taylor, 1992; Gargas and Taylor, 1995; Ogawa et al., 1997; Spatafora, 1995) and *Neolecta* consistently groups with these basal ascomycetes in rDNA analyses (Landvik et al., 1993; Platt and Spatafora, 1997; Platt and Spatafora, 1998).

Bootstrapping was performed to estimate support for clade stability (Hillis and Bull, 1993). Bootstrap values were generated using 1000 replicates of a heuristic search with 10 replicates of random sequence addition and TBR branch swapping. Kishino-Hasegawa maximum-likelihood ratio tests were also performed to provide additional statistical support for the inferred phylogenetic relationships and to test traditional classification concepts of the Geoglossaceae and related taxa. Phylogenetic trees representing null hypotheses of monophyly for various classification concepts were evaluated under the Hasegawa-Kishino-Yano (HKY) (Hasegawa et al., 1985) model of

evolution and compared using the Kishino-Hasegawa (KH) test as implemented in PAUP* (Swofford, 1999). Maximum Likelihood estimations were based on the HKY (1985) model using empirical nucleotide frequencies and starting branch lengths were obtained using Rogers-Swofford approximation method (Swofford, 1999) with no enforcement of a molecular clock. MacClade 3.0.3 (Maddison and Maddison, 1992) was used to create constrained tree topologies representing various classification concepts or null hypotheses. Maximum parsimony analyses utilizing 100 replicates of random sequence addition with TBR branch swapping and gaps treated as newstate were then performed in PAUP* (Swofford, 1999) under the given constraint. In this way, only the most parsimonious trees under a given constraint were compared using the parametric Kishino-Hasegawa maximum likelihood ratio test.

5.4 Results

5.4.1 Combined SSU and LSU rDNA

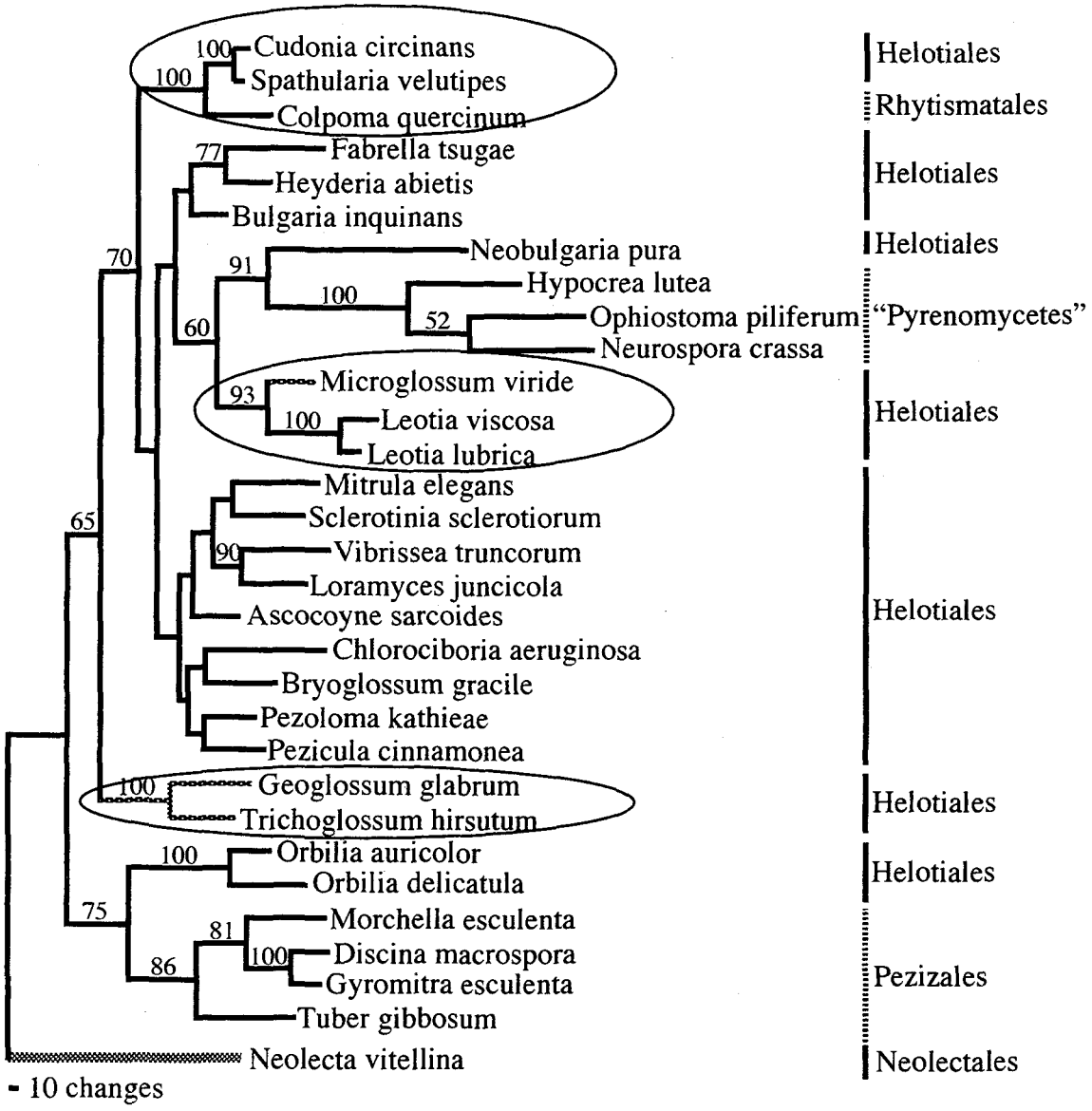
The combined data set included a total of 1664 characters, 369 of which were parsimony informative when gaps were treated as a fifth character state. When gaps were treated as missing data, 352 of the 1664 total characters were parsimony informative. The partition homogeneity test revealed significant incongruence ($P = 0.02000$) however, Cunningham demonstrated that combining data which exhibit partition homogeneity test values of $P > 0.01$ improved or did not decrease phylogenetic accuracy (Cunningham, 1997). Therefore, the SSU and LSU rDNA data were combined. The maximum parsimony heuristic search in which gaps were treated as missing data, found three

equally most parsimonious trees. When gaps were treated as a fifth character state (i.e., newstate) a single most parsimonious tree was found (Figure 5.1). This single most parsimonious tree was identical in topology to the best –ln likelihood tree (Figure 5.1) of the three equally most parsimonious trees from the search in which gaps were treated as missing data (and therefore also had an identical –ln likelihood value of 11345.48965). All trees inferred phylogenies in which the core genera of the Geoglossaceae do not form a monophyletic taxon (see patterned branches in Figure 5.1). *Geoglossum* and *Trichoglossum* appear as sister taxa and form a well-supported monophyletic clade in a relatively basal position of the tree. *Cudonia* and *Spathularia* also appear as well-supported sister taxa but form a clade with *Colpoma quercinum* of the Rhytismatales. *Microglossum* has high bootstrap support as a sister taxon to *Leotia*. Thus, these core genera are distributed relatively evenly throughout the inoperculate discomycetes in these analyses.

5.4.2 Nuclear LSU rDNA

The expanded nuclear LSU rDNA data set representing 40 taxa, half of which are classified in the Geoglossaceae, contained a total of 572 characters, 167 of which were parsimony informative. This data set represents a more thorough sampling of species that are central to familial concepts of the Geoglossaceae. Maximum parsimony analyses were performed under two different sets of character exclusions to accommodate differential treatment of ambiguous regions. The more conservative treatment (i.e., excluding all ambiguous regions) allowed the heuristic search to find 15 equally most parsimonious trees of 800 steps. Including more characters from the ambiguous regions

Figure 5.1 Single most parsimonious tree inferred from maximum parsimony analysis of combined nuclear SSU and LSU rDNA with gaps treated as a fifth character state. This phylogeny has the best –ln likelihood of the three trees that were inferred when gaps were treated as missing data (-ln likelihood = 11345.48965). CI = 0.555 Bootstrap values from 1000 replicates are shown above branches. Higher level classifications are listed to the right of taxon labels. Core "earth tongue" genera are represented with patterned branches. The speckled branch of the outgroup, *Neolecta* denotes a general "earth tongue" fruitbody morphology.



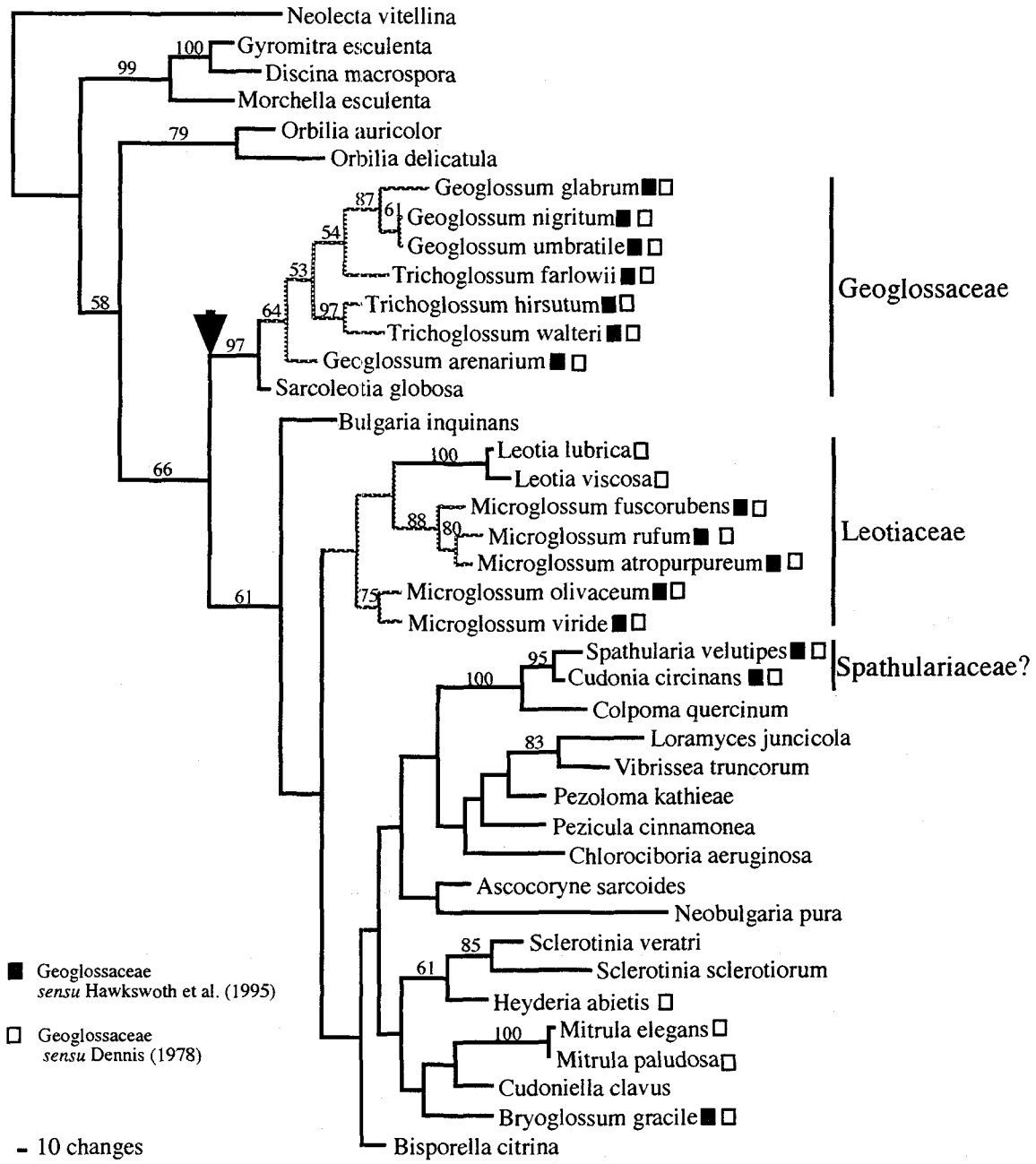
resulted in the heuristic search finding only four equally most parsimonious trees of 800 steps. The best –ln likelihood tree was recovered in both searches under both exclusion classes (Figure 5.2). In all phylogenies the Geoglossaceae did not appear as a monophyletic group. As Figure 5.2 shows, even the generally accepted core genera of the family (i.e., *Geoglossum, Microglossum*, and *Trichoglossum*) are polyphyletic and the Geoglossaceae as currently circumscribed, are a polyphyletic assemblage of taxa. *Geoglossum* appears paraphyletic with *Trichoglossum*, and these two closely related genera form a monophyletic clade. *Sarcoleotia* appears basal to this *Geoglossum/Trichoglossum* clade and together, these three genera form a well-supported monophyletic clade. *Microglossum* also appears paraphyletic, with two distinct, well-supported *Microglossum* clades showing affinity for *Leotia*. *Cudonia* and *Spathularia* appear as well-supported sister taxa, but group with *Colpoma* of the Rhytismatales, rather than with other members of the Geoglossaceae or Helotiales.

Results of the Kishino-Hasegawa maximum likelihood ratio test are shown in Table 5.2. A total of 211 trees representing thirteen sets of equally most parsimonious trees were tested. The monophyly of all previously proposed classification schemes for the Geoglossaceae tested are rejected ($P < 0.0001$) (Table 5.2). The monophyly of the core genera, under this sampling scheme is also rejected ($P = 0.0001 - 0.0002$) (Table 5.2).

5.5 Discussion

The primary goal of this study was to reconstruct the phylogeny of the earth tongues and test the monophyly of the Geoglossaceae using nuclear SSU and LSU rDNA.

Figure 5.2 The best (-ln L – 5090.11331) phylogram of four equally most parsimonious trees of 800 steps inferred from nuclear LSU rDNA data (CI = 0.460). The generally recognized core genera of the Geoglossaceae are shown with patterned branches. Taxa considered to be members of the Geoglossaceae *sensu* Hawksworth et al. (1995) are denoted with black boxes. Taxa classified within the Geoglossaceae *sensu* Dennis (1978) are marked with open boxes. Bootstrap values from 1000 replicates are shown above the respective nodes. The black arrow indicates the suggested, revised monophyletic Geoglossaceae which is united by the presence of brown-walled paraphyses.



The results of the phylogenetic analyses in this study clearly demonstrate that the earth tongue fruitbody morphology (i.e., fleshy and clavate, spathulate or stipitate) does not unite only a single clade of inoperculate discomycetes. Both combined nuclear SSU and LSU rDNA (Figure 5.1) and nuclear LSU rDNA phylogenies (Figure 5.3) reveal that the Geoglossaceae are not a monophyletic taxon (Figure 5.1). Additional statistical support for this non-monophyly is provided with results of the Kishino-Hasegawa test under which two common classification schemes for the Geoglossaceae were tested. When the Hawksworth et al. (Hawksworth et al., 1995) concept of the Geoglossaceae is constrained to monophyly, all equally most parsimonious trees were rejected as alternative hypotheses ($P < 0.0001$) (Table 5.2) to the best tree (Figure 5.3). Furthermore, enforcing this constraint greatly reduced resolution of the inferred phylogeny and the 90 equally most parsimonious trees found under this constraint are 53 steps longer than the best tree found when no constraints were enforced (Table 5.2). Dennis's (1978) concept of the Geoglossaceae faired no better (Table 5.2). Although Dennis's concept resulted in a more stringent comparison than the Hawksworth et al. (1995) hypothesis because of the inclusion of more taxa in his family concept, the resolution increased and only a single most parsimonious tree was found (Table 5.2). However, this tree is 58 steps longer than the best tree (5 steps longer than the Hawksworth et al. set of trees) and the P-value of <0.0001 suggests we must reject his concept of the Geoglossaceae as well.

The Geoglossaceae *sensu* Hawksworth et al. (1995) and *sensu* Dennis (1978) both include the genera *Cudonia* and *Spathularia*. In all phylogenies inferred in this study, these genera were distant from other members of the Geoglossaceae and sister to *Colpoma* of the Rhytismatales (Figure 5.1 – 5.3), which is consistent with other findings

Table 5.2 Results of the Kishino-Hasegawa Maximum Likelihood ratio test. The number of trees refers to the number of equally most parsimonious trees found after a heuristic search under the given constraint. Length (in steps) refers to the parsimony score, or number of steps in the set of equally most parsimonious trees.

Hypothesis of Monophyly	# of trees	Length (in steps)	Range of –ln L	Range of P-values	Results
Unconstrained,	4	800	5090.11331	1.0	Best
Fewer excluded characters			5105.03350	0.2998	
Unconstrained,	15	800	5090.11331	1.0	Best
More excluded characters			5100.17018	0.2988	
Geoglossaceae sensu	90	853	5309.22027	<0.0001	Reject
Hawksworth et al. (1995)			5324.64837		
Geoglossaceae sensu Dennis (1977)	1	858	5336.92113	<0.0001	Reject
Geoglossum, Microglossum	23	825	5196.31084	0.0002	Reject
& Trichoglossum			5204.87842	<0.0001	
Geoglossum, Microglossum	10	817	5165.24717	0.0027	Reject
Trichoglossum & Leotia			5174.68814	0.0005	
Geoglossum	4	801	5098.84871	0.2956	
			5101.17001	0.2603	
Microglossum	36	803	5099.98009	0.6088	
			5109.66883	0.3581	
M. atropurpureum in	4	836	5234.00763	<0.0001	Reject
Geoglossum clade			5236.48362		
G. arenarium in	5	819	5176.23576	0.0021	Reject
Microglossum clade			5182.70062	0.0005	
Sarcoleotia globosa in	2	817	5153.90390	0.0016	Reject
Leotia clade			5156.03137	0.0015	
Leotiaceae sensu	5	846	5306.45637	<0.0001	Reject
Hawksworth et al. (1995)			5309.32836		
Leotiaceae sensu	12	836	5254.41099	<0.0001	Reject
Dennis (1977)			5261.44712		

based on SSU rDNA (Gernandt, 1998; Gernandt et al., 1998; Platt and Spatafora, 1997; Platt and Spatafora, 1998). This relationship, which has excellent bootstrap support in these analyses (Figure 5.1 & 5.3), is difficult to compare with macromorphological characters as *Colpoma* and many other members of the Rhytismatales produce fairly small ascomata that are generally immersed or erumpant from plant tissue. These

relatively reduced ascomata of the Rhytismatales are in stark contrast to the large, fleshy spathulate and stipitate ascomata of *Cudonia* and *Spathularia*. However, perhaps the shared characters of elongated, hyaline spores and curved tips of the paraphyses represent synapomorphies reflecting a common evolutionary origin. The placement of *Cudonia, Spathularia,* and *Colpoma* point to a major problem remaining in inoperculate discomycete systematics; that is defining the phylogenetic boundary between the Helotiales and Rhytismatales (Gernandt, 1998; Korf, 1990; Platt and Spatafora, 1998). Regardless of this ordinal boundary, however, these data clearly show that *Cudonia* and *Spathularia* are not closely related to *Geoglossum* and are not best accommodated in the Geoglossaceae. Future studies should address whether *Cudonia* and *Spathularia* should be given ordinal recognition (i.e., Spathulariales) or whether they are better placed in the Rhytismatales.

An important finding of this study is that even the generally accepted core genera of the Geoglossaceae (i.e., *Geoglossum, Microglossum,* and *Trichoglossum*) do not form a monophyletic group based on SSU and LSU rDNA data (Figures 5.1 & 5.3). Although there have been conflicts regarding the generic limits within the family (Korf, 1990), these genera have always been treated as central to the family concept. These data show that *Microglossum* is not closely related to *Geoglossum* and *Trichoglossum* , but is more closely related to *Leotia* (Figures 5.1 & 5.3). The sister relationship of *Microglossum* with *Leotia* is supported with a high bootstrap value (i.e., 93) in the combined data set (Figure 5.1) but is not supported by bootstrap in the LSU rDNA data set (Table 5.3). The Kishino-Hasegawa test also suggests that *Microglossum* is not closely related to *Geoglossum* and *Trichoglossum* (Table 5.2). The two different null hypotheses placing

Microglossum as a close relative of *Geoglossum* and *Trichoglossum* which were tested were both rejected under the maximum likelihood ratio test (Kishino and Hasegawa, 1989). In the test which constrained *Geoglossum, Trichoglossum*, and *Microglossum* to monophyly, 23 equally most parsimonious trees were found which were 25 steps longer (Table 5.2) than the best tree (Figure 5.3). The parametric P-value ranged between 0.0002 and < 0.0001 which indicates we must reject these "core" genera as being monophyletic. In a more liberal approach, *Leotia* was included as a member of the monophyletic clade with *Geoglossum, Trichoglossum,* and *Microglossum*. The heuristic search under this topological constraint, found ten equally most parsimonious trees and therefore showed increased resolution over the previous constraint of *Geoglossum, Trichoglossum,* and *Microglossum* (Table 5.2). This set of ten equally most parsimonious trees was 17 steps longer than the best tree (Figure 5.3) but eight steps shorter than the previous constraint which did not include *Leotia* (Table 5.2). The range of maximum-likelihood ratio p-values was also slightly better than under the previous constraint ($P = 0.0027 - 0.0005$) but still fell well below the $P < 0.05$ cut-off. Therefore, the Kishino-Hasegawa test shows that we must reject the hypothesis that *Microglossum* is close relative of *Geoglossum* and *Trichoglossum* and should not be considered a core genus of the Geoglossaceae.

The phylogenetic position of *Microglossum* inferred in these analyses is noteworthy since some familial classifications have recognized separate treatments for the "brown-spored" and the "white-spored Geoglossaceae" (Nannfeldt, 1942; Maas Geesteranus, 1964). Furthermore, the affinity of *Microglossum* for *Leotia* in these analyses points again to problems of some earth tongue genera traversing the "nebulous

borderland between the Geoglossaceae and Helotiaceae" (Maas Geesteranus, 1966). These analyses support the opinions of Korf (Korf, 1958) and Maas Geesteranus (Maas Geesteranus, 1964) that *Leotia* is better accommodated in the Helotiaceae (Leotiaceae) than in the Geoglossaceae, however they provide strong evidence that *Microglossum* should also be removed from the family Geoglossaceae. The Kishino-Hasegawa test was implemented to help statistically define the phylogenetic boundary between the Geoglossaceae (as currently circumscribed) and the Leotiaceae, specifically with respect to the position of *Microglossum* . Concepts of the Leotiaceae *sensu* Hawksworth et al. (1995) and *sensu* Dennis (1978) were tested by constraint to monophyly. Constrained trees representing both null hypotheses were rejected in favor of the best tree ($P <$ 0.0001) (Table 5.2) suggesting that neither classification accurately describes a monophyletic group. The delimitation of *Leotia* and *Microglossum* was also tested by constraining the paraphyletic *Microglossum* (see Figure 5.3) to monophyly. Maximum parsimony analyses under this constraint of a monophyletic *Microglossum* found 36 trees which were only three steps longer than the best tree shown in Figure 5.3 (Table 5.2). The p-values of 0.06088 – 0.3581 were well above the acceptable cut-off of $P < 0.05$, suggesting that a monophyletic *Microglossum* can not be rejected (Kishino and Hasegawa, 1989). Although the best LSU rDNA phylogeny (Figure 5.3) suggests that *Microglossum* and *Leotia* may not be well-defined, monophyletic genera, results of the KH test show there is no statistical evidence for changing the current generic limits.

In light of historical subfamilial classifications (Nannfeldt, 1942) (Maas Geesteranus, 1964), this observed phylogenetic position of *Microglossum* outside of the Geoglossaceae now begs the question, "Are there any white-spored members of the

Geoglossaceae?". This study addresses that question with the taxon sampling of the nuclear LSU rDNA data set. Figure 5.3 shows that *Sarcoleotia globosa* and *Geoglossum arenarium*, which have hyaline (or white) spores, are basal members of the clade comprised of *Geoglossum* and *Trichoglossum*. *Geoglossum arenarium* is a synonym of *Microglossum arenarium* and this species has occupied several generic positions over the years (i.e., *Corynetes*, Durand, 1908 (Durand, 1908) and *Thuemenidium,* (Maas Geesteranus, 1964). This study recognizes *G. arenarium* as the most appropriate name to be used here given this taxon sampling scheme. Although admittedly, using the generic name *Geoglossum* infers paraphyly for the genus in the phylogeny presented (Figure 5.3). The generic confusion regarding *G. arenarium* seems to be centered around the fact that this species has hyaline spores (i.e., "white-spored Geoglossaceae"), thus warranting it's treatment in *Microglossum* by some authors (Imai, 1941;Saccardo, 1894). Results of the Kishino-Hasegawa (KH) test show that *G. arenarium* should not be considered a member of *Microglossum*. (Table 5.2). Maximum parsimony analyses constraining *G. arenarium* within the *Microglossum* and *Leotia* clade resulted in 5 equally most parsimonious trees that were 19 steps longer than the best tree (Figure 5.3 & Table 5.2). The maximum likelihood ratio test resulted in p-values of 0.0021 – 0.0005, suggesting that the null hypothesis of *G. arenarium* as a member of the *Microglossum* clade, must be rejected. This finding is notable since *Microglossum atropurpureum*, which is well-supported within the *Microglossum* clade (Figure 5.3), has also been considered a synonym of *Geoglossum* (Fries, 1821) and *Thuemenidium* (Maas Geesteranus, 1964). This disparity is contrary to previous hypotheses that these two species are closely related and members of the same genus, *Thuemenidium* Maas

Geesteranus, 1964 #4188]. The Kishino-Hasegawa (KH) test was also used to provide statistical evidence for this disparate relationship (Table 5.2). In maximum parsimony analyses in which *M. atropurpureum* is constrained within the *Geoglossum* and *Trichoglossum* clade, four equally most parsimonious trees were found that were 36 steps longer than the best tree (Figure 5.3) The KH test resulted in a p-value of < 0.0001 which suggests the placement of *M. atropurpureum* in the *Geoglossum* clade must be rejected (Table 5.2).

Sarcoleotia globosa, which is placed in a basal position relative to *Geoglossum arenarium* (Figure 5.3), also has hyaline spores. This taxon has typically been treated as a member of the Helotiaceae (Leotiaceae), although character similarities with members of the Geoglossaceae have been recognized (Maas Geesteranus, 1966). These analyses suggest that *Sarcoleotia globosa* is a close relative of *Geoglossum* and *Trichoglossum* and is therefore probably best accommodated in the Geoglossaceae rather than the Leotiaceae. Results of the KH test also support this hypothesis and show that the concept of *S. globosa* in the *Leotia* and *Microglossum* clade must be rejected ($P < 0.05$, $P =$ 0.0016 - 0.0015) (Table 5.2). Therefore, these data infer and support the hypothesis that there are "white-spored" (i.e., hyaline-spored) members of the Geoglossaceae, as long as the concept of the Geoglossaceae is modified to include *Geoglossum arenarium* and *Sarcoleotia globosa*. The characters of spore color and morphology, traditionally used in these classifications, do not appear to be good phylogenetic characters at this level. Neither brown spores nor white spores represent syanapomorphies for the Geoglossaceae, however, all members of this clade possess brown-walled paraphyses and this character appears to be synapomorphic.

As previously mentioned, *Geoglossum* appears as paraphyletic assemblage in the LSU rDNA phylogeny due to the placement of *Trichoglossum* and *G. arenarium*. (Figure 5.3). Preliminary phylogenetic studies based on nuclear LSU, ITS, and Group I introns have also inferred a paraphyletic *Geoglossum* (Zhong et al., 1999). The monophyly of *Geoglossum* was tested in this study using the parametric KH test and found to not be significantly worse than the paraphyly inferred in the best tree (Table 5.2). Therefore, no statistical evidence exists for making nomenclatural changes to deal with position of *G. arenarium* relative to other *Geoglossum* species. Additional taxon and character sampling within this group should help elucidate whether or not future taxonomic changes are needed with regard to generic delimitation of *Geoglossum*.

The phylogenies presented in this study suggest that of the genera traditionally classified within the Geoglossaceae, only *Geoglossum* and *Trichoglossum* should be retained. However, modern concepts of the Geoglossaceae should be expanded to include *Sarcoleotia* and therefore, the family is still recognized to be comprised of hyaline-spored species (i.e., *Sarcoleotia* and *G. arenarium*). This *noveau* concept of the Geoglossaceae is based primarily on cladistic analyses of DNA data from a single locus, the nuclear rDNA repeat unit and should be tested and refined in future studies using data from multiple loci and a greater taxon sampling. However, this "Geoglossaceae" clade is supported with a high bootstrap value (Figure 5.3) and the statistics from the parametric KH test (Table 5.2). In addition, as previously mentioned, this clade of "Geoglossaceae" is united by the presence of brown-walled paraphyses, a character lacking in *Microglossum* and other members of the Geoglossaceae *sensu lato*.

This reconstruction of the earth tongue phylogeny based on nuclear SSU and LSU rDNA data, has demonstrated that the Geoglossaceae as currently circumscribed, is not monophyletic. The earth tongue fruitbody morphology is present in several different distantly related lineages of ascomycetes. Landvik (1996) used phylogenetic analysis of nuclear SSU and LSU rDNA from *Neolecta* to show that fruit-bodies exist in the most basal lineage of the ascomycetes (Archiascomycetes). Previously however, *Neolecta* had been classified in the Geoglossaceae (Korf, 1973). Use of *Neolecta* as an outgroup in these analyses raises the ponderance of the primitive form of fruitbody in the ascomycetes. But, convergent evolution of fruitbody morphology could also explain the presence of "earth tongues" in so many, relatively unrelated lineages. This convergent evolutionary pattern has been demonstrated in the hypogeous fungi (i.e., truffle-like fungi) and could be a factor in these phylogenies as well (Bruns et al., 1989). Many questions still remain regarding the evolution of earth tongues, not the least of which is the generic, familial, and even ordinal limits for these amazing and diverse inoperculate discomycetes. This study has addressed a first step in this myriad of questions by utilizing the "suitable characters" of nucleotide sequence data to better define the Geoglossaceae and work toward a more natural classification of the "unwieldy Leotiaceae".

5.6 Acknowledgements

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CHAPTER 6

Phylogenetic Hypotheses for the Inoperculate Discomycetes Inferred from Multiple Loci Sequence Data

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6.1 Abstract

Sequence analyses of nuclear SSU rDNA, combined nuclear SSU and LSU rDNA, and RNA Polymerase II (RPB2) sequence data were used to compare inferred phylogenies for the inoperculate discomycetes (Ascomycota, Euascomycetes, Helotiales). The congruence of some consistently observed relationships constitute reciprocal corroboration of accurate phylogenetic hypotheses for some major groups of inoperculate discomycetes. These data show that the Helotiales are not a monophyletic group, rather members of this order are a polyphyletic assemblage with diverse life history strategies. Furthermore, the earth tongue morphology, which has been used to unite members of the Geoglossaceae, is found in at least three distinct clades of the Ascomycota, thus rejecting the monophyly of the Geoglossaceae. Enigmatically, *Cudonia* and *Spathularia* are more closely related to members of the Rhytismatales than they are to members of the Geoglossaceae or other members of the Helotiales. In addition, disparate relationships between other ascomycetes exhibiting a general earth tongue fruitbody morphology expose a pattern of convergent evolution in this discomycete ascomal morphology. The inclusion of *Neolecta* in these analyses and its placement basal to the Euascomycetes, supports paraphyses as being a synapomorphy for the Euascomycetes. However, the characters of ascogenous hyphae and ascomata production may be symplesiomorphic for the Euascomycetes as they are present in a more basal group of the Ascomycota. The number of lichenization events among the Euascomycetes is still unclear as are other evolutionary strategies that may have arisen early in the evolutionary history of these fungi

6.2 Introduction

Most recent phylogenetic hypotheses for the higher level classification of the Ascomycota are based on nucleotide sequence data from nuclear small subunit ribosomal DNA (SSU rDNA) (Berbee and Taylor, 1992; Kurtzman, 1993; Nishida and Sugiyama, 1993). These data infer three major lineages of the Ascomycota; the Archiascomycetes, Saccharomycetes, and Euascomycetes. The Euascomycetes are generally united by production of ascogenous hyphae and formation of an ascocarp (Alexopoulos et al., 1996). However, a basal lineage of the Ascomycota, the Neolectales, also produces an ascocarp but lacks paraphyses. Understanding the evolution of ascomycetes is further complicated by the fact that the fungi of the Euascomycetes exhibit a broad range of ascomata and ascus morphologies, leading to the hypothesis that a radiation event occurred closely associated with the origin of the Euascomycete lineage. Consistent with this hypothesis, molecular phylogenetic studies using nuclear SSU rDNA sequence data have resulted in trees with unstable placement of "backbone" nodes and lack of consensus regarding super ordinal classification (Berbee, 1996; Spatafora, 1995). The unstable "backbone" can be further characterized by well supported terminal clades but poorly supported (both in number and consistency of characters) internal clades. It is noted, however, that a phylogenetic pattern consistent with a radiation event is not a test of such an event (Spatafora, 1995).

Despite the inability of the SSU rDNA data to resolve the Euascomycete "backbone", the data do strongly resolve some of the major terminal clades of the class and generally infer seven major lineages within the Euascomycetes: the "Plectomycetes", three orders of "Loculoascomycetes (*ie.*, Chaetothyriales, Pleosporales, and Dothideales),

the "Pyrenomycetes", the operculate "Discomycetes" and the inoperculate "Discomycetes" (Berbee, 1996; Spatafora, 1995). The term "Discomycete" is commonly used to describe those Euascomycetes which have ascohymenial development resulting in an open apothecium, and unitunicate asci, *ie.*, asci with a single functional wall layer (Hawksworth et al., 1995). The operculate discomycetes are differentiated from the inoperculate discomycetes by having asci which forcibly discharge their spores through a specialized lid, *ie.*, operculum near the tip of the ascus rather than simply through a pore or slit, *ie.*, inoperculate asci.

The inoperculate discomycetes, represented primarily by the Helotiales and Rhytismatales, have been under-represented in the taxon sampling of several studies, although the former represents one of the largest orders of ascomycetes (Berbee and Taylor, 1995; Gargas et al., 1995; Gargas and Taylor, 1995; Spatafora, 1995). This paucity of taxon sampling for this group of fungi has led to phylogenies which infer monophyly for the inoperculate discomycetes. This "artifactual" monophyly is contradictory to hypotheses that the Helotiales are paraphyletic at best (Korf, 1990; Pfister, 1997). Recently, several studies have focused on increased taxon sampling of the inoperculate discomycetes in an effort to more accurately test and refine phylogenetic hypotheses for the Euascomycetes (Gernandt et al., 1998; Platt and Spatafora, in review; Platt and Spatafora, 1998). These analyses all support the rejection of the monophyly for both orders, Helotiales and Rhytismatales. However, the quality of inference of large phylogenies has also been debated due to the potential loss of phylogenetic accuracy (Kim, 1996). Despite the controversy surrounding recovery of an accurate, large phylogeny, several recent studies suggest that increased, directed taxon sampling can

actually improve the accuracy of maximum parsimony-based phylogenies (Graybeal, 1998; Hillis, 1996; Rannala et al., 1998).

The problem of poor resolution and lack of support along the Euascomycete "backbone" in parsimony-based SSU rDNA phylogenies is most likely attributable to the paucity of parsimony informative characters supporting internal nodes. Increasing the taxon-sampling in such data sets does not improve resolution or support because increasing the number of taxa causes a decrease in the number of informative characters per taxon (Graybeal, 1998). This observation is suggestive that increasing character sampling coupled with increased taxon sampling would improve phylogenetic resolution and accuracy of large phylogenies (Cummmings et al., 1995; Hillis et al., 1994; Lecointre et al., 1994; Lecointre et al., 1993). Thus, character sampling is another factor critical in construction of robust phylogenetic hypotheses for the Euascomycetes. While nuclear SSU rDNA has served as a good starting point for modern phylogenetic hypotheses, the number of characters this gene region provides is not sufficient to overcome the problems of poor topological resolution, lack of character support and increased taxon sampling within the Ascomycota (Berbee et al., 1998). One proposed solution in rDNA-based phylogenetic studies is to increase character sampling with additional nucleotide characters from other regions of the rDNA repeat. The nuclear LSU rDNA region, although generally perceived to evolve faster than SSU rDNA, contains conserved domains in which the nucleotide variation is comparable to that of SSU rDNA. Several recent fungal phylogenetic studies have incorporated this principle of increasing rDNA characters by combining SSU and LSU rDNA data (Gams et al., 1998; Holst-Jensen et al., 1997; Johnson, 1999; Spatafora et al., 1998).

The principle of congruence in phylogenetic systematics is based on agreement among trees derived from independent sources of data which constitutes strong reciprocal corroboration of accurate phylogenetic inference (De Queiroz et al., 1995; Hillis, 1995; Miyamoto and Fitch, 1995). This form of "total evidence" approach is contingent upon availability of independent data which is problematic in higher level ascomycete systematics because the vast majority of nucleotide data currently available is derived from SSU rDNA. However, a recent study has shown that *RPB2*, the gene coding for the second largest subunit of RNA polymerase II, infers phylogenies similar to those from SSU rDNA data for the major lineages of ascomycetes (Liu et al., in press (1999)). Parsimony analyses of this protein-coding, single copy nuclear gene inferred topologies that were largely consistent with those from parsimony analyses of the SSU rDNA, differing only at weakly supported nodes (Liu et al., in press (1999)). However, limited representation of the inoperculate discomycetes in the RPB2 sampling, inferred a phylogeny in which inoperculate discomycetes form a monophyletic clade.

The objective of this study was to test and refine phylogenetic hypotheses for the Euascomycetes with emphases on the systematics of the inoperculate discomycetes, evolution of ascomal morphologies and phylogenetic origins of the lichen symbiosis. Our approach utilized phylogenetic analyses of nuclear SSU and LSU rDNA and *RPB2* sequence data and compared phylogenetic hypotheses across three independent data sets.

6.3 Materials and Methods

6.3.1 Taxon Sampling

A total of 266 taxa, representing some 42 families of inoperculate discomycetes were sampled. Three criteria were used to select exemplar taxa: i) familial classification, ii) ascomal morphology and iii) life history. A comprehensive list of the taxa sampled in this study is provided in Table 6.1.

Table 6.1 Taxa sampled in this study. The ordinal and familial classifications used partially follows that of Hawksworth et al. 1995. GenBank Accession numbers are given for those sequences available on GenBank. An asterisk (*) denotes those sequences determined by the first author as part of this study. Sequences determined in other laboratories which are not yet available on GenBank are identified with the appropriate initials. (AH= Arne Holst-Jensen, DG=Dave Gernandt, FL=Francois Lutzoni, JJ = Jacqui Johnson, JS = Joseph Spatafora, SK =Scott Kroken)

Classification	Taxon	GenBank SSU	GenBank LSU	GenBank RPB2
BASIDIOMYCOTA	*Agaricus bisporus*	NA	NA	AF107785
	Athelia bombacina Pers.	M55638	NA	NA
	Dictyonema pavonia	U23541	NA	NA
	Spongipellis unicolor	M59760	NA	NA
ASCOMYCOTA				
ARCHIASCOMYCETES				
Schizosaccharomycetales	*Schizosaccharomyces pombe*	NA	NA	D13337
INCERTAE SEDIS				
Neolectales				
Neolectaceae	*Neolecta irregularis*	Z47721	NA	NA
	Neolecta vitellina	Z27393	Z48318	AF107786
	Neolecta vitellina	*	*	NA
HEMIASCOMYCETES				
Saccharomycetales				
Saccharomycetaceae	*Candida albicans*	X53497	X70659	AF107787
	Candida krusei	NA	NA	AF107788
	Kluyveromyces lactis	X51830	NA	NA
	Saccharomyces cerevisiae	V01335	J01355	M15693
	Zygosaccharomyces rouxii	X58057	NA	NA
EUASCOMYCETES				
"Pyrenomycetes"				
Arthoniales				

Table 6.1 (Continued)

Arthoniaceae	*Arthonia radiata*	U23537	NA	NA
Roccellaceae	*Dendrographa leucophaea*	U23538	NA	NA
	Lecanactis abietina	U23539	NA	NA
	Schismatomma pericleum	U23540	NA	NA
Diaporthales				
Valsaceae	*Leucostoma persoonii*	M83259	NA	NA
	Cryphonectria radicalis	L42442	NA	NA
Hypocreales				
Hypocreaceae	*Hypocrea lutea*	D14407	U00739	NA
	Hypomyces chrysospermus	M89993	NA	NA
Clavicipitaceae	*Claviceps paspali*	U32401	NA	NA
Ophiostomatales				
Ophiostomataceae	*Ophiostoma ulmi*	M83261	NA	NA
	Ophiostoma stenoceras	M85054	NA	NA
	Ophiostoma piliferum	U20377	U47837	NA
Microascales				
Microascaceae	*Microascus cirrosus*	M89994	NA	NA
	Pseudallescheria boydii	M89782	NA	NA
Sordariales				
Sordariaceae	*Neurospora crassa*	X04971	U40124	AF107789
Chaetomiaceae	*Chaetomium elatum*	M83257	NA	AF107791
Lasiosphaeriaceae	*Podospora anserina*	NA	NA	AF107790
Verrucariales				
Verrucariaceae	*Dermatocarpon ler luridum*	JW	NA	NA
	Verrucaria pacifica	JW	NA	NA
Xylariales				
Xylariaceae	*Xylaria carpophi*	Z49785	NA	NA
	Daldinia concentrica	U32402	NA	NA
"Plectomycetes"				
Erysiphales				
Erysiphaceae	*Blumeria graminis*	L26253	NA	NA
	Phyllactinia guttata	AF021796	NA	NA
Eurotiales				
Trichocomaceae	*Penicillium notatum*	M55628	M55628	NA
	Aspergillus fumigatus	M55626	U28460	NA
	Aspergillus nidulans	NA	NA	AF107793
	Byssochlamys nivea	NA	NA	AF107794
	Eurotium rubrum	U00970	NA	NA
	Talaromyces flavus	M83262	NA	NA
	Trichophyton rubrum	NA	NA	AF107795
Onygenales				
Onygenaceae	*Ajellomyces capsulatus*	S45469	NA	NA
	Blastomyces dermatitidis	M55624	NA	NA
	Coccidiodes immitis	M55627	NA	NA
	Geomyces pannorum	AB016174	NA	NA
	Histoplasma capsulatum	X58572	NA	NA
"Loculoascomycetes"				
Chaetothyriales				
Herpotrichiellaceae	*Capronia pilosella*	U42473	NA	AF107798
	Capronia mansonii	NA	NA	AF107797
mitosporic	*Exophiala dermatitidis*	X79312	NA	NA
	Exophiala mansonii	X78480	NA	NA
	Exophiala jeanselmei	NA	NA	AF107796

Table 6.1 (Continued)

	Fonsecaea pedrosoi	L36997	NA	NA
	Phialophora verrucosa	L36999	NA	NA
Dothideales				
Dothideaceae	*Septoria nodorum*	U04236	NA	NA
mitosporic	*Aureobasidium pullulans*	NA	NA	AF107799
	Dothidea hippophaeos	U42475	NA	NA
Lophiostomataceae	*Lophiostoma crenatum*	U42485	NA	NA
	Lophiostoma caulium	AF164362	U43469	NA
	Dothidea insculpta	U42474	NA	AF107800
Pleosporales				
Pleosporaceae	*Pleospora herbarum*	U05201	U43476	AF107804
anamorphic	*Stemphylium botryosum*	NA	NA	AF107804
	Pleospora rudis	U00975	NA	NA
	Alternaria alternata	U05194	NA	NA
	Cochliobolus sativus	U42479	NA	AF107803
anamorphic	*Curvularia brachyspora*	NA	NA	AF107803
	Pyrenophora trichostoma	U43459	U43477	NA
	Pyrenophora tritici-repentis	U42486	NA	NA
Mycosphaerellaceae	*Mycosphaerella citrullina*	NA	NA	AF107801
Leptosphaeriaceae	*Leptosphaeria bicolor*	U04202	NA	NA
	Leptosphaeria doliolum	U43447	U43475	NA
Sporormiaceae	*Sporormia lignicola*	U42478	NA	NA
	Sporormiella minima	NA	NA	AF107805
Botryosphaeriaceae	*Botryosphaeria rhondina*	U42476	NA	AF107802
	Botryosphaeria ribis	U42477	NA	NA
"Discomycetes"				
Cyttariales				
Cyttariaceae	*Cyttaria darwinii*	U53369	NA	NA
Elaphomycetales				
Elaphomycetaceae	*Elaphomyces aculeatus*	U45439	NA	NA
	Elaphomyces leveillei	U45441	NA	NA
Gyalectales				
Gyalectaceae	*Gyalecta ulmi*	AF088237	NA	NA
Helotiales				
Baeomycetaceae	*Baeomyces placophyllus*	AF107349*	AF107560*	NA
	Baeomyces rufus	AF107348*	AF107559*	NA
	Baeomyces rufus	AF107347*	AF107558*	NA
Dermateaceae	*Pezicula cinnamonea*	*	*	*
	Podophacidium xanthomelum	*	NA	NA
Geoglossaceae	*Geoglossum arenarium*	NA	*	NA
	Geoglossum glabrum	*	AF113738*	*
	Geoglossum nigritum	*	*	AF107806*
	Geoglossum umbratile	NA	*	NA
	Sarcoleotia globosa	*	*	NA
	Trichoglossum farlowii	NA	*	NA
	Trichoglossum hirsutum	*	*	NA
	Trichoglossum walteri	NA	*	NA
Hemiphacidiaceae	*Fabrella tsugae*	DG	*	NA
	Hemiphacidium longisporum	DG	NA	NA
	Meria laricis	DG	NA	NA
	Rhabdocline parkeri	DG	NA	NA
	Sarcotrochila macrospora	DG	NA	NA
Hyaloscyphaceae	*Arachnopeziza aurata*	U67427	NA	NA

Table 6.1 (Continued)

	Lachnellula calyciformis	U67431	NA	NA
	Lachnellula tenuissimus	NA	*	NA
	Lachnum virgeneanus	JJ	JJ	NA
	Dasyscyphus tenuissimus	NA	*	NA
Hymenoscyphaceae	*Hymenoscyphus fructigens*	U67430	NA	NA
	Hymenoscyphus caudatus	JJ	JJ	NA
	Hymenoscyphus conscriptus	JJ	JJ	NA
	Hymenoscyphus virgulatorum	Z81382	Z81410	NA
Icmadophilaceae	*Dibaeis absolutus*	NA	AF113731*	NA
	Dibaeis baeomyces	AF113712*	AF113730*	*
	Dibaeis baeomyces	AF113713*	AF107555*	NA
	Dibaeis baeomyces	*	NA	NA
	Dibaeis baeomyces	JW	NA	NA
	Icmadophila ericetorum	AF113709*	AF107556*	*
	Siphula ceratites	U72712	AF107557*	NA
	Siphula coriacea	*	AF113724*	NA
	Siphula pickeringii	NA	AF113727*	NA
	Siphula polyschides	AF113710*	AF113725*	*
	Thamnolia subuliformis	AF113714*	AF113733*	*
	Thamnolia vermicularis	NA	AF113732*	NA
Leotiaceae	*Ascocoryne sarcoides*	*	*	NA
	Bisporella citrina	NA	*	NA
	Bryoglossum gracile	*	*	NA
	Bulgaria inquinans	AJ224362	*	NA
	Chlorociboria aeruginosa	*	*	NA
	Chlorociboria aeruginosa	AH	AH	NA
	Chloroscypha chloromela	DG	NA	NA
	Chloroscypha enterochroma	DG	NA	NA
	Chloroscypha seaveri	DG	NA	NA
	Cudoniella clavus	NA	*	*
	Encoelia fascicularis	Z81379	NA	NA
	Gremmeniella abietina	DG	NA	NA
	Heyderia abietis	*	*	NA
	Leotia lubrica	L37536	*	NA
	Leotia viscosa	*	*	AF107807
	Microglossum atropurpureum	NA	*	NA
	Microglossum fuscorubens	NA	*	NA
	Microglossum olivaceum	NA	*	NA
	Microglossum rufum	NA	*	NA
	Microglossum viride	*	*	AF107806*
	Microglossum viride	U46031	NA	NA
	Neobulgaria premnofila	U45444	NA	NA
	Neobulgaria pura	U45444	*	NA
	Pezoloma kathieae	*	*	*
	Stamnaria americana?	*	*	NA
Loramycetaceae	*Loramyces juncicola*	DG	*	*
Orbiliaceae	*Orbilia auricolor*	U72598	*	*
	Orbilia delicatula	U72603	*	*
Phacidiaceae	*Phacidium coniferarum*	DG	NA	NA
	Phacidium infestans	DG	NA	NA
Rustroemiaceae	*Rustroemia firma*	Z81395	NA	NA
	Rustroemia firma	*	NA	NA
	Sclerotinia homeocarpa	Z81397	Z81421	NA

Table 6.1 (Continued)

Sclerotiniaceae	*Botryotinia convoluta*	HJ	HJ	NA
	Botrytis cinerea	HJ	HJ	NA
	Ciboria amantacea	JJ	JJ	NA
	Ciboria caucus	HJ	HJ	NA
	Ciborinia foliicola	Z81376	Z81404	NA
	Cibornina whetzellii	HJ	HJ	NA
	Gloeotinia granigena	Z81380	Z81408	NA
	Gloeotinia sp.	JJ	JJ	NA
	Lambertella langei	DG	JJ	NA
	Mitrula elegans	*	*	*
	Mitrula paludosa	NA	*	NA
	Monilinia fruticola	HJ	HJ	NA
	Monilinia laxa	Y14210	Z73752	NA
	Monilinia oxycocci	Z73727	Z73754	NA
	Piceomphale bulgarioides	Z81388	Z81415	NA
	Pycnopeziza sympodialis	HJ	HJ	NA
	Sclerotinia sclerotiorum	X69850	AJ226089	AF107808
	Sclerotinia veratri	NA	AF113739*	NA
Vibrisseaceae	*Vibrissea truncorum*	*	*	NA
incertae sedis	*Cudonia circinans*	AF107343*	*	*
	Cudonia confusa	Z30240	NA	NA
	Spathularia flavida	Z30239	NA	NA
	Spathularia velutipes	AF107344*	AF107554*	NA
Lecanorales				
Acarosporaceae	*Acarospora schleicheri*	AF088236	NA	NA
Bacidiaceae	*Squamarina lentigera*	AF088250	NA	NA
Cladoniaceae	*Cladia aggregata*	U72713	NA	NA
	Cladonia bellidiflora	U60900	NA	NA
	Cladonia coniocraea	JW	NA	NA
	Cladonia subsquamosa	JW	NA	NA
Lecanoraceae	*Lecanora dispersa*	L37535	NA	NA
Lecidiaceae	*Lecidea fuscoatra*	AF088239	NA	NA
Megalosporaceae	*Megalospora sulphurata*	AF08824	NA	NA
Parmeliaceae	*Evernia prunastri*	*	*	NA
	Letharia vulpina	SK	NA	NA
	Pseudevernia cladoniae	AF088245	NA	NA
Physciaceae	*Heterodermia hypoleuca*	FL	FL	NA
	Physcia aipolia	AF088243	NA	NA
	Santessonia namibensis	AF088248*	NA	NA
Porpidiaceae	*Porpidia crustulata*	L37540	NA	NA
Rhizocarpaceae	*Rhizocarpon geographicum*	AF088246	NA	NA
	Rhizocarpon geographicum	JW	NA	NA
Rimulariaceae	*Rimularia fuscosora*	AF088247	NA	NA
Stereocaulaceae	*Pilophorus acicularis*	U70960	NA	NA
	Stereocaulon paschale	FL	FL	NA
	Stereocaulon ramulosum	U70961	NA	NA
	Stereocaulon tomentosum	JW	NA	NA
Trapeliaceae	*Placopsis perrugosa*	FL	FL	NA
Umbilicariaceae	*Lasallia papulosum*	JW	NA	NA
	Lasallia pennsylvanica	JW	NA	NA
	Lasallia rossica	AF088238	NA	NA
	Umbilicaria rigida	JW	NA	NA
	Umbilicaria subglabra	AF088253	NA	NA

Table 6.1 (Continued)

Ostropales				
Graphidaceae	*Graphis scripta*	AF038878	NA	NA
Stictidaceae	*Conotrema populorum*	U86582	NA	NA
	Cyanodermella virdula	U86583	NA	NA
	Stictis radiata	U20610	*	NA
Thelotremataceae	*Diploschistes ocellatus*	AF038876	NA	NA
Peltigerales				
Lobariaceae	*Densdrisocaulon intricatulum*	JW	JW	NA
	Lobaria quercizans	JW	NA	NA
Nephromataceae	*Nephroma arcticum*	X89219	NA	NA
Peltigeraceae	*Hydrothyria venosa*	JW	NA	NA
	Peltigera canina	JW	NA	NA
	Peltigera neopolydactyla	X89219	NA	NA
	Solorian crocea	X89220	NA	NA
Pertusariales				
Megasporaceae	*Megaspora verrucosa*	AF088241	NA	NA
Pertusariaceae	*Ochrolechia frigida*	FL	FL	NA
	Pertusaria amara	FL	FL	NA
	Pertusaria saximontana	*	NA	NA
	Pertusaria trachythallina	AF088242	NA	NA
incertae sedis	*Loxosporopsis corallifera*	*	*	*
Pezizales				
Balsamiaceae	*Balsamia vulgaris*	AF054905	NA	NA
Helvellaceae	*Discina macrospora*	U42651	U42678	NA
	Gyromitra esculenta	U42648	U42675	NA
	Helvella lacunosa	U53378	NA	NA
	Rhizina undulata	U42664	NA	NA
Morchellaceae	*Morchella esculenta*	U42642	U42669	NA
	Morchella elata	U42641	U42667	AF107810
Otidiaceae	*Inermisia aggregata*	Z30241	NA	NA
Pezizaceae	*Peziza badia*	L37539	NA	NA
	Peziza quelepidotia	(U42665)	(U42693)	AF107809
	Peziza succosa	U53383	NA	NA
Sarcoscyphaceae	*Sarcoscypha austriaca*	U53392	NA	NA
Sarcosomataceae	*Plectania nigrella*	Z27408	NA	NA
Terfeziaceae	*Terfezia arenaria*	AF054898	NA	NA
Thelebolaceae	*Thelebolus crustaceus*	U53394	NA	NA
Tuberaceae	*Tuber gibbosum*	U42663	U42690	NA
Rhytismatales				
Ryhtismataceae	*Coccomyces farlowii*	*	NA	NA
	Colpoma quercinum	DG	*	*
	Cyclaneusma minus	DG	NA	NA
	Elytroderma deformans	DG	NA	NA
	Lirula macrospora	DG	NA	NA
	Lophodermium pinastri	DG	NA	NA
	Lophodermium sp.	DG	NA	NA
	Meloderma desmazierii	DG	NA	NA
	Naemacyclus fimbriatus	DG	NA	NA
	Pseudographis elatina	DG	NA	NA
	Rhytisma acerinum?	*?	*?	NA
	Rhytisma salicinum	U53370	NA	NA
	Tryblidiopsis pinastri	DG	NA	NA
incertae sedis	*Darkera parca*	DG	NA	NA

Table 6.1 (Continued)

Teloschistales				
Teloschistaceae	*Teloschistes chrysophtalmus*	AF088252	NA	NA
	Xanthoria elegans	AF088254	NA	NA
"Other"				
Caliciales				
Caliciaceae	*Cyphelium inquinans*	U86695	NA	NA
	Texosporium sancti-jacobi	U86696	NA	NA
	Thelomma mammosum	U86697	NA	NA
	Calicium adspersum?	U86694	NA	NA
Mycocaliciaceae	*Chaenothecopsis savonica*	U86691	NA	NA
	Mycocalicium albonigrum	L37538	NA	NA
	Stenocybe pullatula	U86692	NA	NA
	Sphinctrina turbinata	U86693	NA	NA
Sphaerophoraceae	*Sphaerophorus globosus*	L37532	NA	NA
	Sphaerophorus globosus	FL	FL	NA
	Bunodophoron scrobiculatum	U70958	NA	NA
	Leifidium tenerum	U70959	NA	NA

6.3.2 Character Sampling

Total genomic DNA was extracted from fungal specimens following the methods described previously (Platt and Spatafora, in review; Platt and Spatafora, 1999, in press). These studies also outline the conditions used for amplification of nuclear SSU and LSU rDNA via polymerase chain reaction (PCR) (Platt and Spatafora, in review; Platt and Spatafora, 1999, in press). Approximately 1 kb of the 3'end of the *RPB2* gene was amplified using the primers fRPB2-7cR and fRPB2-11aR (Liu et al., in press (1999)). The polymerase chain reaction cycling parameters were 94°C for 3 minutes, 35 cycles of 94°C for 1 minute, 51°C for 30 seconds, and 72°C for 1 minute, followed by 5 cycles of 94°C for 1 minute, 53°C for 30 seconds, then 72°C for 1 minute plus 5 seconds per cycle, with a final 5 minute 72°C incubation. All amplicons were purified prior to

sequencing reactions using an ammonium acetate precipitation (Platt and Spatafora, 1999, in press). Nucleotide sequences were determined on either an ABI 373 or 377 automated DNA sequencer by the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University. The primers used for sequencing reactions were either those outlined in previous studies (Platt and Spatafora, in review; Platt and Spatafora, 1999, in press) or, as in the case of the *RPB2* locus, were the same as those used for PCR amplification.

6.3.3 Phylogenetic Analyses

Contig assembly of the nuclear SSU and LSU rDNA sequences followed methods described previously (Platt and Spatafora, in review; Platt and Spatafora, 1999, in press). The nucleotide sequences from *RPB2* were edited and converted into predicted amino acid sequences using EditView 1.0.1 (Applied Biosystems, Inc.). These partial amino acid sequences were then added to a larger data set of complete *RPB2* amino acid sequence data (Liu et al., in press (1999)). In both rDNA data sets and the protein-coding data set of RPB2, regions with length heterogeneity or ambiguously alignable characters, were excluded from subsequent parsimony analyses. Alignments are available through the TreeBase web site: http://www.herbaria.harvard.edu/treebase.

Phylogenetic analyses were conducted using PAUP* 4.0b2a (Swofford, 1999). The nuclear SSU rDNA and combined nuclear SSU and LSU rDNA data sets were analyzed using maximum parsimony. Heuristic searches of unweighted SSU rDNA data were conducted using 10 replications of random sequence addition with Tree-bisection-reconnection (TBR) branch swapping. Due to the large number of taxa included in the

SSU rDNA data set and constraints on computational requirements, an upper limit of 2000 trees (max trees = 2000) was set.

A nuclear SSU and LSU rDNA data set was assembled and the combinability of the two rDNA regions was assessed using the partition homogeneity test (100 replicates, random sequence addition and TBR branch swapping) (Swofford, 1999). Sets of most parsimonious trees from the rDNA analyses were compared under the HKY maximum likelihood model of evolution (Hasegawa et al., 1985).

Maximum parsimony and weighted parsimony were used in analyses of the predicted amino acid data from *RPB2* in order to test previously inferred RPB2-based phylogenetic hypotheses for the ascomycetes. Maximum parsimony heuristic search options were the same as those applied in the rDNA analyses, however, no constraint was placed on the number of maximum trees. Two weighted parsimony analyses of the RPB2 data set were conducted, successive approximation (Farris, 1969) and implementation of a JTT step matrix (Felsenstein, 1981; Jones et al., 1992). The same JTT step matrix which was used in the first RPB2-inferred phylogeny of ascomycetes (Liu et al., in press) was used in this study to test the consistency of these protein-coding based hypotheses.

6.4 Results and Discussion

6.4.1 Nuclear SSU rDNA

Maximum parsimony analyses of SSU rDNA data from 203 ingroup taxa, were based on a total of 1182 characters, 462 of which were parsimony-informative. The maximum of 2000 trees was retained in the heuristic searches; these trees were 5831

steps with a consistency index (CI) of 0.310. All trees had identical placement of the root between *Neolecta* and the rest of the Ascomycetes. The tree from the entire set of 2000 most parsimonious trees (mpts) with the best –ln likelihood is shown in Figure 6.1. All trees reflected phylogenies which infer that the Helotiales are a polyphyletic assemblage of taxa and that the discomycete morphology is symplesiomorphic for the euascomycetes. The phylogeny inferred from maximum parsimony analysis of nuclear SSU rDNA agrees with previous studies with respect to placement of the root for ascomycetes (Gargas et al., 1995; Gernandt, 1998; Landvik, 1996). Using the Basidiomycota – the sister group to the Ascomycota – as an outgroup, the root to the Ascomycota was placed between *Neolecta* (Archiascomycetes) and the Saccharomycetes and Euascomycetes (Figure 6.1). The nuclear SSU rDNA phylogeny inferred (Figure 6.1) is consistent with other SSU rDNA based studies which suggest that the Pezizales or operculate discomycetes are the most basal lineage of euascomycetes (Gernandt, 1998) (Platt, Chapter 5)(Gargas et al., 1995). This inference further implies that the discomycete form of ascomata, *ie.*, the apothecium, is primitive or symplesiomorphic for the Euascomycetes. The analyses of increased taxon sampling, especially with respect to the inoperculate discomycetes, rejects the monophyly of the order Helotiales (Figure 6.1). Furthermore, the observed topology is consistent with the hypothesis that inoperculate asci are primitive morphologies from which the other ascal types found within the Euascomycetes have been derived. These results and those from previous studies based on SSU rDNA reject the monophyly of the Geoglossaceae *sensu* Hawksworth and Dennis. All analyses are consistent with a pattern convergent evolution of the earth tongue morphology inferring up to four independent gains within the Ascomycota. The number of lichenization events within the

Figure 6.1 The best –ln likelihood tree of 2000 equally most parsimonious trees inferred from a heuristic search of 462 parsimony-informative characters from SSU rDNA nucleotide sequence data. A schematic of the phylogram backbone is provided in the upper left-hand corner for orientation (I). The strict consensus cladogram (II) shows relative resolution. Part A refers to the basal portion of the phylogram, with Part B showing relationships within the more derived lineages. Bootstrap values from 10 replicates of a full heuristic search using random sequence addition and TBR branch swapping are shown above respective nodes. Lineages to lichenized taxa are represented with patterned branches. The major groups of ascomycetes based on ascomatal morphology is given beside terminal nodes; "Loculo" = "Loculoascomycetes", "Pyren" = "Pyrenomycetes", "Plect" = "Plectomycetes", with ordinal classifications given for the "Discomycetes". The "Discomycete" orders represented are Lec = Lecanorales, Pert = Pertusariales, Ela = Elaphomycetales, Tel = Teloschistales, Pel = Peltigerales, Ost = Ostropales, Helo = Helotiales, Pez = Pezizales, Rhy = Rhytismatales, and Cytt = Cyttariales. The lichenized orders Verrucariales (Ver; "Pyrenomycetes") and Caliciales (Cal) are also designated.

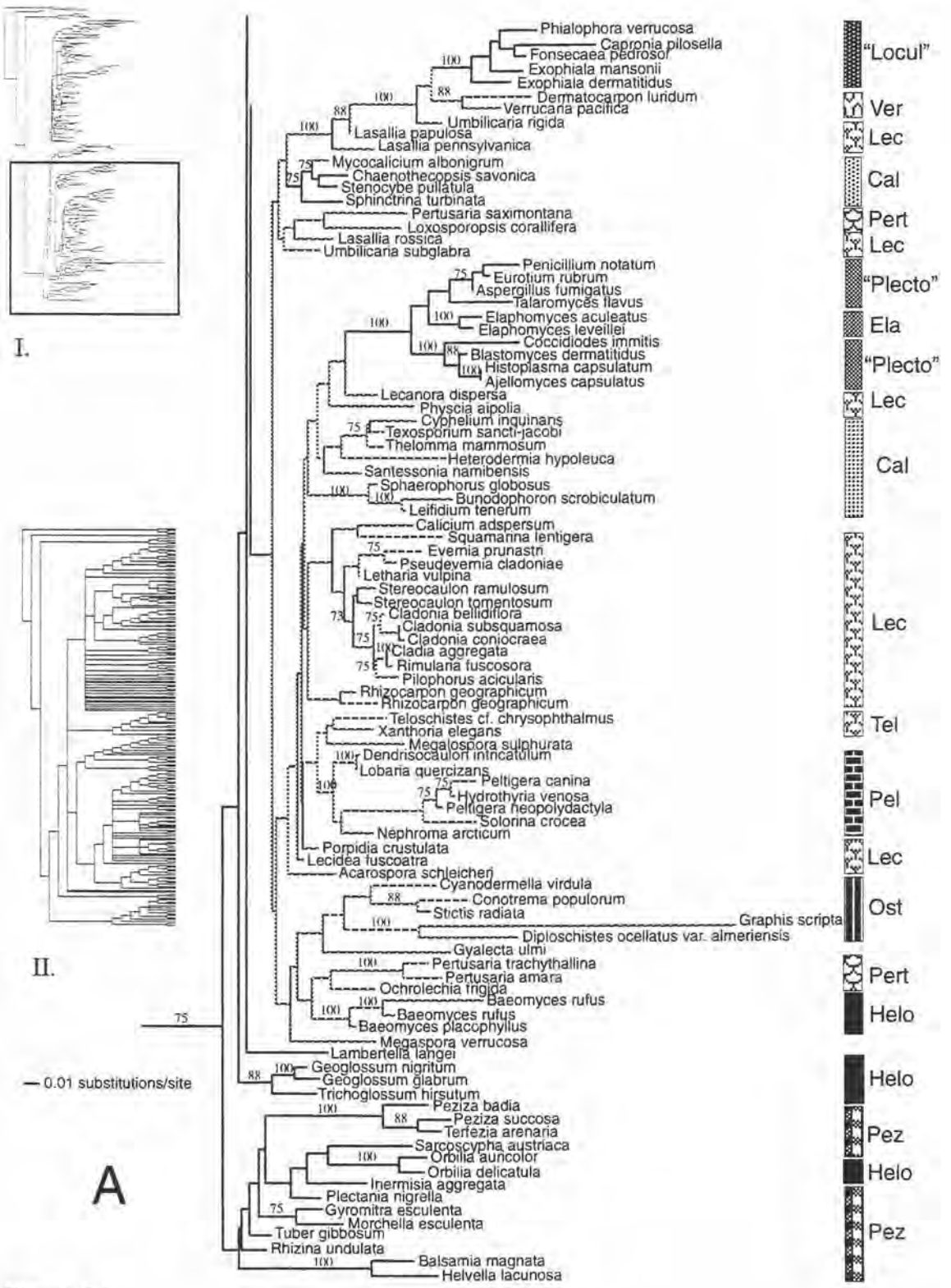

I.
II.
A
— 0.01 substitutions/site
Phialophora verrucosa
Capronia pilosella
Fonsecaea pedrosoi
Exophiala mansonii
Exophiala dermatitidus
Dermatocarpon luridum
Verrucaria pacifica
Umbilicaria rigida
Lasallia papulosa
Lasallia pennsylvanica
Mycocalicium albonigrum
Chaenothecopsis savonica
Stenocybe pullatula
Sphinctrina turbinata
Pertusaria saximontana
Loxosporopsis corallifera
Lasallia rossica
Umbilicaria subglabra
Penicillium notatum
Eurotium rubrum
Aspergillus fumigatus
Talaromyces flavus
Elaphomyces aculeatus
Elaphomyces leveillei
Coccidiodes immitis
Blastomyces dermatitidus
Histoplasma capsulatum
Ajellomyces capsulatus
Lecanora dispersa
Physcia aipolia
Cyphelium inquinans
Texosporium sancti-jacobi
Thelomma mammosum
Heterodermia hypoleuca
Santessonia namibensis
Sphaerophorus globosus
Bunodophoron scrobiculatum
Leifidium tenerum
Calicium adspersum
Squamarina lentigera
Evernia prunastri
Pseudevernia cladoniae
Letharia vulpina
Stereocaulon ramulosum
Stereocaulon tomentosum
Cladonia bellidiflora
Cladonia subsquamosa
Cladonia coniocraea
Cladia aggregata
Rimularia fuscosora
Pilophorus acicularis
Rhizocarpon geographicum
Rhizocarpon geographicum
Teloschistes cf. chrysophthalmus
Xanthoria elegans
Megalospora sulphurata
Dendrisocaulon intricatulum
Lobaria quercizans
Peltigera canina
Hydrothyria venosa
Peltigera neopolydactyla
Solorina crocea
Nephroma arcticum
Porpidia crustulata
Lecidea fuscoatra
Acarospora schleicheri
Cyanodermella virdula
Conotrema populorum
Stictis radiata
Graphis scripta
Diploschistes ocellatus var. almeriensis
Gyalecta ulmi
Pertusaria trachythallina
Pertusaria amara
Ochrolechia frigida
Baeomyces rufus
Baeomyces rufus
Baeomyces placophyllus
Megaspora verrucosa
Lambertella langei
Geoglossum nigritum
Geoglossum glabrum
Trichoglossum hirsutum
Peziza badia
Peziza succosa
Terfezia arenaria
Sarcoscypha austriaca
Orbilia auricolor
Orbilia delicatula
Inermisia aggregata
Plectania nigrella
Gyromitra esculenta
Morchella esculenta
Tuber gibbosum
Rhizina undulata
Balsamia magnata
Helvella lacunosa
"Locul"
Ver
Lec
Cal
Pert
Lec
"Plecto"
Ela
"Plecto"
Lec
Cal
Lec
Tel
Pel
Lec
Ost
Pert
Helo
Helo
Pez
Helo
Pez

Figure 6.1

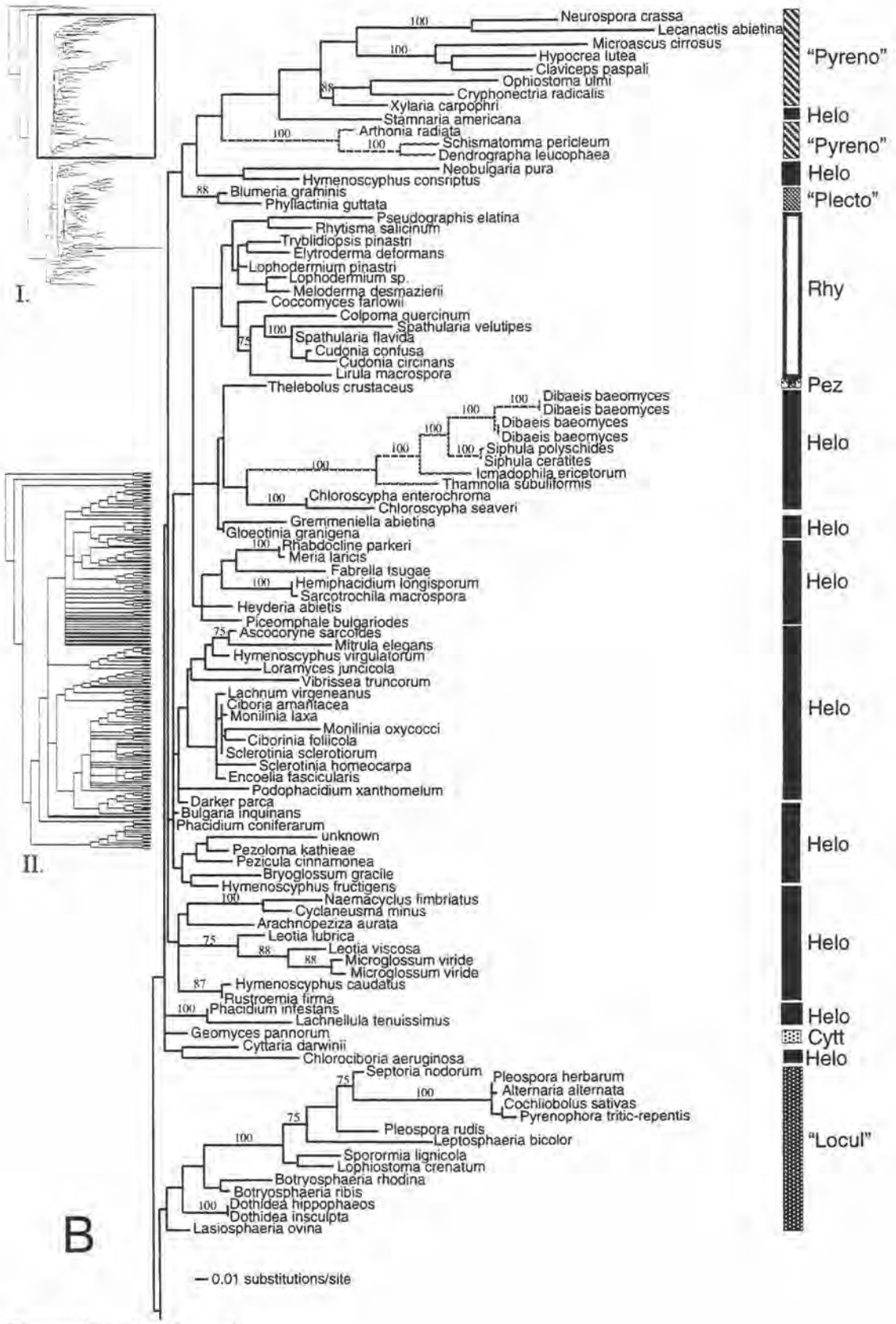


Figure 6.1 (Continued)

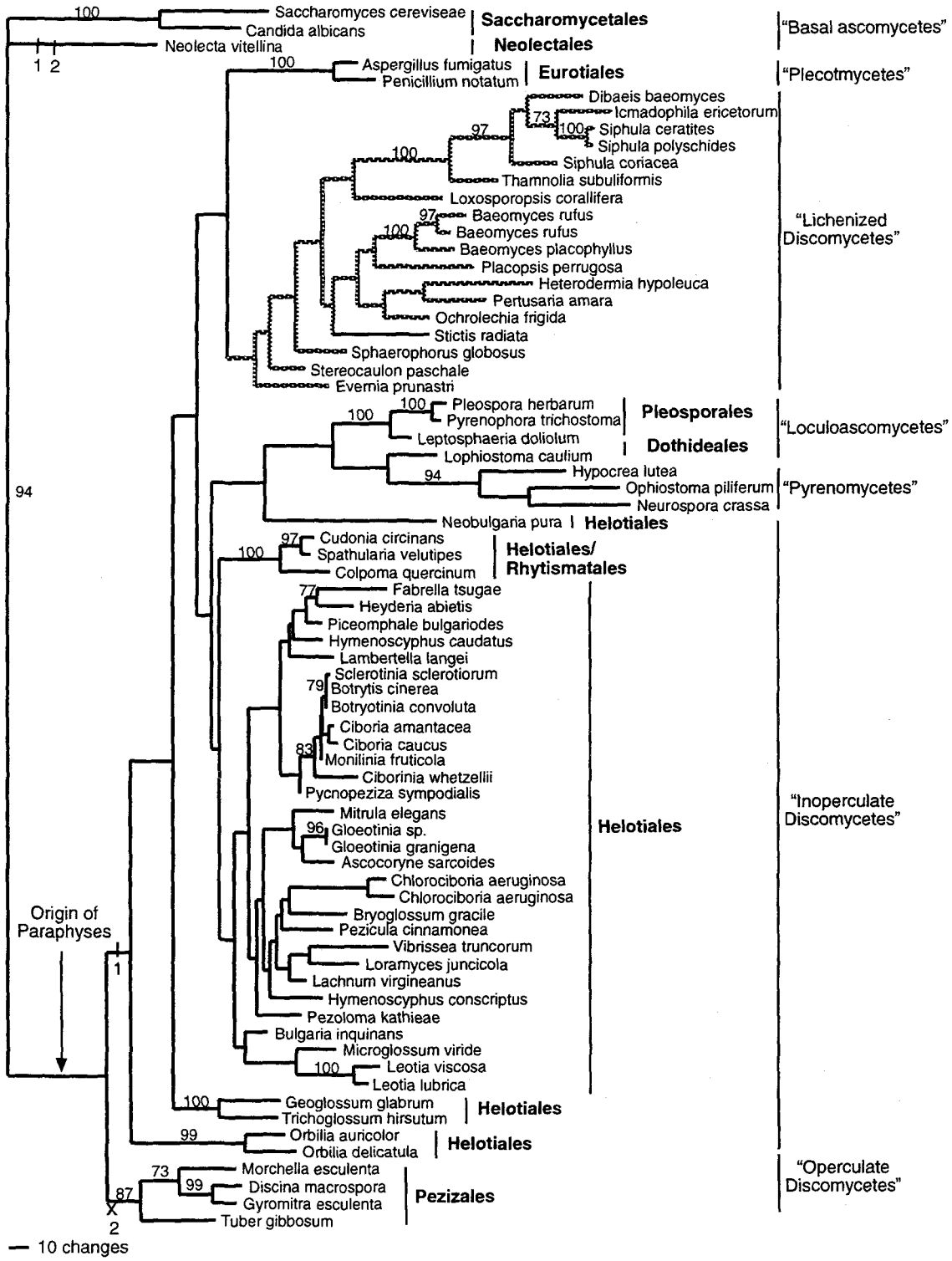
euascomycetes is not completely resolved with the sampling of this study. Multiple origins of the lichen symbiosis are hypothesized, both for the Ascomycota and Basidiomycota (Gargas et al., 1995; Lutzoni and Vilgalys, 1995) however, polarizing characters to determine whether certain fungal lineages represent losses or gains of lichenization is equivocal. The large taxon sampling of this study, which provides a good representation of some previously under-sampled lichenized groups did not improve resolution enough to successfully polarize the character of lichenization.

This observation may be attributed to the nature of nuclear SSU rDNA which simply does not provide enough informative characters at the short nodes, hypothesized to be the result of a rapid radiation event (Spatafora, 1995). It seems logical that a rapid radiation event could lead to diversification of life history strategies, such as lichen and endophytic symbioses and so recovering the origins of these patterns is especially problematic.

6.4.2 Combined Nuclear SSU and LSU rDNA

The partition homogeneity test (Farris et al., 1994; Mickevich and Farris, 1981) of the nuclear SSU and LSU data partitions yielded a P-value of 0.0100, a value found to be within the acceptable limits for combining data from different partitions (Cunningham, 1997) and so the data were combined. The combined data set was comprised of a total of 1708 characters from 72 taxa. Sixty equally most parsimonious trees of 2505 steps were found which had a CI of 0.324. The best –ln likelihood tree under the HKY model of evolution (Hasegawa et al., 1985) is shown in Figure 6.2. As in the nuclear SSU rDNA inferred phylogeny, the Helotiales appear as a polyphyletic taxon and the general discomycete morphology is symplesiomorphic for the Euascomycetes. Also in

Figure 6.2 Best –ln likelihood tree of 60 equally most parsimonious trees inferred from combined nuclear SSU and LSU rDNA data. Bootstrap values from 100 replicates are indicated above branches. Two alternative hypotheses regarding inoperculate asci are presented: 1 = two independent gains, 2 = a single gain followed by a single loss



agreement with the nuclear SSU rDNA phylogeny (Figure 6.1) is the basal placement of the Pezizales and *Orbilia* (Helotiales) (Figure 6.2).

The combined analyses of the SSU and LSU rDNA focused on examplar sampling of major Euascomycete clades. This increase in characters sampled is in contrast to the original SSU rDNA analyses which are characterized by increased taxon sampling. Reduced taxon sampling in the combined rDNA data set was necessary due to missing LSU rDNA data for many of the taxa included in the original SSU rDNA analyses. However, by utilizing exemplar taxon sampling we attempted to avoid problems associated with inadequate representation of various lineages. The combined data set inferred a phylogeny very similar to that of the SSU rDNA in recognition of the major monophyletic clades (Figure 6.2), although one stark contrast in the phylogenetic hypotheses presented by the two data sets is the number of lichenization events. The dense taxon sampling of the SSU rDNA data set leads to an inference of a minimum of three gains of the lichen symbiosis followed by several subsequent losses within smaller lineages (Figure 6.1). Contrary to this hypothesis of convergence on the lichen habit, the combined SSU and LSU rDNA data set infers only a single gain of lichenization (Figure 6.2). The difference between these two estimates of the number of gains and losses of lichenization stems largely from both the ambiguous placement of the two lichenized families within this order, the Baeomycetaceae and Icmadophilaceae (Fig. 6.1 and 6.2) and the absence of several groups of lichenized fungi, (e.g., the Arthoniales, Caliciales, Verrucariales etc.) in the combined analyses (Fig. 6.2). However, combining the nuclear SSU and LSU data adds support to several hypotheses inferred from SSU rDNA data alone. The position of the primary lineages of inoperculate discomycetes is relatively

consistent. First, the basal placement of *Orbilia* infers that this inoperculate discomycete is more closely related to the operculate discomycetes than it is to most other members of the Helotiales (Figure 6.2). Next, members of the Geoglossaceae, *Geoglossum* and *Trichoglossum*, also appear to be relatively basal Euascomycetes and are not inferred to be closely related to other members of the Helotiales (Figures 6.1 and 6.2). Finally, the inference of a primary Helotiales clade, centered around some members of the Leotiaceae and Sclerotiniaceae emerges, but the relationships within this group are primarily still unresolved and not highly supported (Figures 6.1 and 6.2).

6.4.3 RNA Polymerase II

Maximum parsimony analysis of the RPB2 amino acid data resulted in 151 equally most parsimonious trees of 2734 steps (CI = 0.593). The strict consensus cladogram shows that the inoperculate discomycetes (*ie.*, *Orbilia*) are inferred to be the most basal lineage of Euascomycetes (Figure 6.3A). Successive approximation analyses based on the rescaled consistency index inferred similar topologies (Figure 6.3B). Thirty four equally most parsimonious trees were recovered from a single round of successive approximation; a second round of successive approximation converged on the same trees. The weighted parsimony analysis using the 250 PAM JTT step matrix resulted in a single most parsimonious tree (Figure 6.3C).

The phylogenies inferred through maximum parsimony and weighted parsimony analyses of RPB2 share some striking topological congruencies with the phylogenies inferred with rDNA data but generally result in an alternate placement of the root (Figure

Figure 6.3 Results of maximum parsimony and weighted analyses of the RPB2 amino acid sequence data. A.) The strict consensus cladogram of 151 equally most parsimonious trees inferred from maximum parsimony analyses. B.) The strict consensus cladogram of 34 equally most parsimonious trees inferred from a single round of successive approximation weighting. C.) The single most parsimonious tree inferred from implementation of a JTT step matrix in weighted parsimony. Lichenized lineages in the phylogram (Tree C) are shown with patterned branches. General higher level classifications are given to the right of terminal nodes.

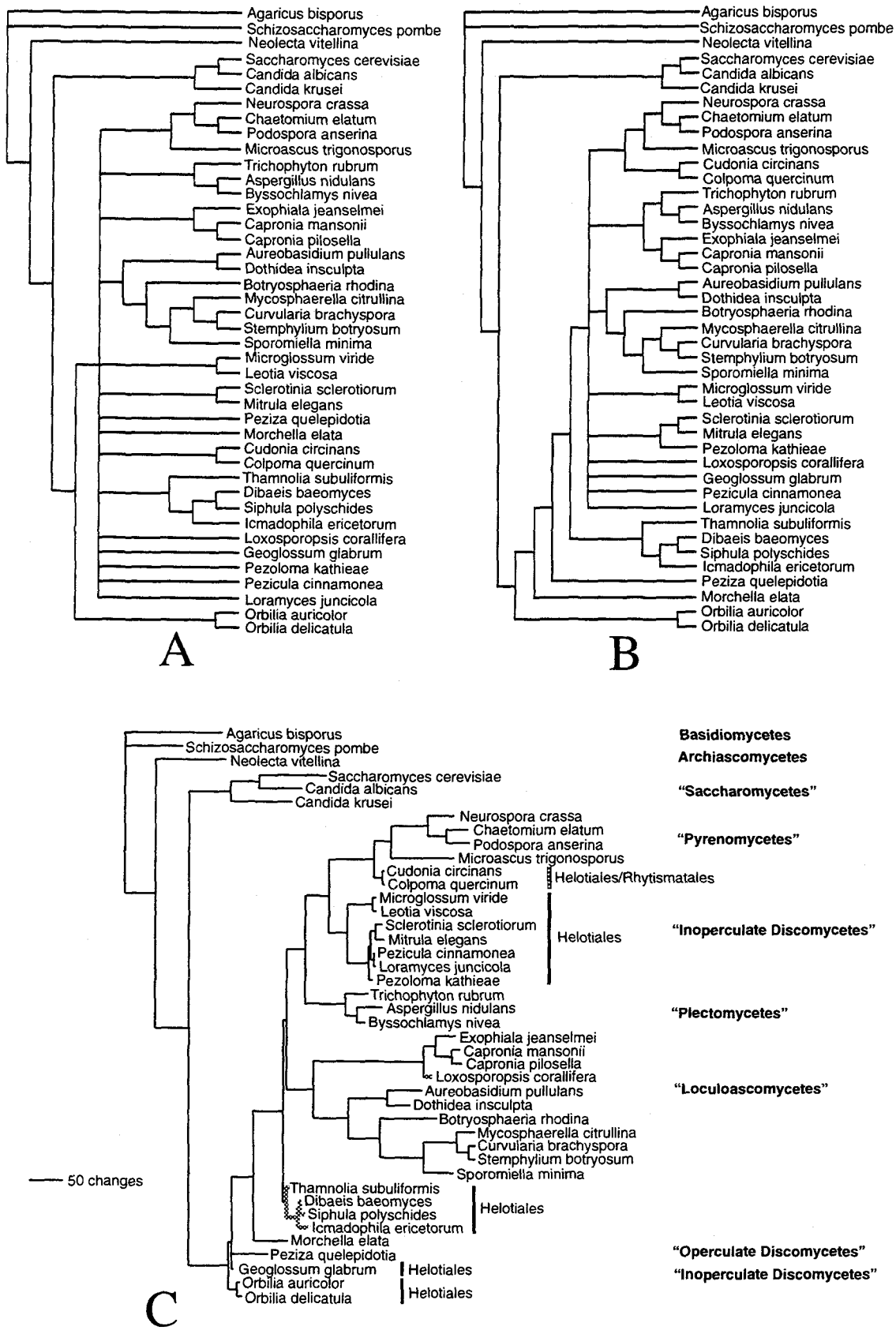

Agaricus bisporus
Schizosaccharomyces pombe
Neolecta vitellina
Saccharomyces cerevisiae
Candida albicans
Candida krusei
Neurospora crassa
Chaetomium elatum
Podospora anserina
Microascus trigonosporus
Trichophyton rubrum
Aspergillus nidulans
Byssochlamys nivea
Exophiala jeanselmei
Capronia mansonii
Capronia pilosella
Aureobasidium pullulans
Dothidea insculpta
Botryosphaeria rhodina
Mycosphaerella citrullina
Curvularia brachyspora
Stemphylium botryosum
Sporormiella minima
Microglossum viride
Leotia viscosa
Sclerotinia sclerotiorum
Mitrula elegans
Peziza quelepidotia
Morchella elata
Cudonia circinans
Colpoma quercinum
Thamnolia subuliformis
Dibaeis baeomyces
Siphula polyschides
Icmadophila ericetorum
Loxosporopsis corallifera
Geoglossum glabrum
Pezoloma kathieae
Pezicula cinnamonea
Loramyces juncicola
Orbilia auricolor
Orbilia delicatula
A
Agaricus bisporus
Schizosaccharomyces pombe
Neolecta vitellina
Saccharomyces cerevisiae
Candida albicans
Candida krusei
Neurospora crassa
Chaetomium elatum
Podospora anserina
Microascus trigonosporus
Cudonia circinans
Colpoma quercinum
Trichophyton rubrum
Aspergillus nidulans
Byssochlamys nivea
Exophiala jeanselmei
Capronia mansonii
Capronia pilosella
Aureobasidium pullulans
Dothidea insculpta
Botryosphaeria rhodina
Mycosphaerella citrullina
Curvularia brachyspora
Stemphylium botryosum
Sporormiella minima
Microglossum viride
Leotia viscosa
Sclerotinia sclerotiorum
Mitrula elegans
Pezoloma kathieae
Loxosporopsis corallifera
Geoglossum glabrum
Pezicula cinnamonea
Loramyces juncicola
Thamnolia subuliformis
Dibaeis baeomyces
Siphula polyschides
Icmadophila ericetorum
Peziza quelepidotia
Morchella elata
Orbilia auricolor
Orbilia delicatula
B
Agaricus bisporus
Schizosaccharomyces pombe
Neolecta vitellina
Saccharomyces cerevisiae
Candida albicans
Candida krusei
Neurospora crassa
Chaetomium elatum
Podospora anserina
Microascus trigonosporus
Cudonia circinans
Colpoma quercinum
Microglossum viride
Leotia viscosa
Sclerotinia sclerotiorum
Mitrula elegans
Pezicula cinnamonea
Loramyces juncicola
Pezoloma kathieae
Trichophyton rubrum
Aspergillus nidulans
Byssochlamys nivea
Exophiala jeanselmei
Capronia mansonii
Capronia pilosella
Loxosporopsis corallifera
Aureobasidium pullulans
Dothidea insculpta
Botryosphaeria rhodina
Mycosphaerella citrullina
Curvularia brachyspora
Stemphylium botryosum
Sporormiella minima
50 changes
Thamnolia subuliformis
Dibaeis baeomyces
Siphula polyschides
Icmadophila ericetorum
Morchella elata
Peziza quelepidotia
Geoglossum glabrum
Orbilia auricolor
Orbilia delicatula
Helotiales/Rhytismatales
Helotiales
Helotiales
Helotiales
Basidiomycetes
Archiascomycetes
"Saccharomycetes"
"Pyrenomycetes"
"Inoperculate Discomycetes"
"Plectomycetes"
"Loculoascomycetes"
"Operculate Discomycetes"
"Inoperculate Discomycetes"
C

Figure 6.3

6.3). All RPB2 analyses placed the root of the euascomycetes between *Orbilia* and the rest of the filamentous ascomycetes (Figure 6.3). The inferred basal position of the Pezizales was lost in maximum parsimony analyses due to the lack of resolution at the node just internal to the root (Figure 6.3A). The weighted parsimony analysis utilizing successive approximation increased resolution of the data set and placed the Pezizales in a relatively basal position, however, not as a monophyletic taxon (Figure 6.3B). In this analysis, the Pezizales are intermediate in the grade between *Orbilia* and the rest of the Euascomycetes (Figure 6.3B). Implementing the JTT step matrix in weighted parsimony greatly improved resolution and yielded a single most parsimonious tree (Figure 6.3C). This phylogeny infers that *Geoglossum* is the closest relative of *Orbilia* and that it is more basal than the Pezizales. Again, the Pezizales are not inferred to be a monophyletic taxon.

6.5 Conclusions

In these analyses both the Helotiales and Rhytismatales, as currently circumscribed (Hawksworth et al., 1995), do not represent monophyletic lineages. Furthermore, the phylogenetic boundary between these two orders is clouded by the intrusion of Rhytismatales in some consistent Helotialean clades, and likewise, the intrusion of Helotialean taxa into some Rhytismatalean clades. In addition to the non-monophyly of the inoperculate discomycetes (Helotiales and Rhytismatales), the "earth tongue" fruitbody morphology of the Helotiales is apparently an artifact of convergent evolution and is observed in at least four independent ascomycete lineages. One of the most provocative relationships is that of *Cudonia* (and *Spathularia*) with members of the

Rhytismatales. Macromorphological characters do not predict this phylogenetic relationship, especially since *Cudonia* and *Spathularia* have the overall earth tongue morphology, producing relatively large, fleshy ascocarps, while many members of the Rhytismatales are endophytes producing small apothecia which are typically immersed or erumpant but generally not fleshy. SSU rDNA phylogenies have inferred this unpredicted relationship before (Gernandt, 1998; Gernandt et al., 1998; Platt and Spatafora, 1998) but because single gene phylogenies alone, can be misleading, there was cause to question the accuracy of this inference. However, this study provides additional data and corroborative evidence from two independent loci and three gene regions to suggest that this may indeed represent the true phylogeny of these inoperculate discomycetes and some ascomatal morphologies may be even more prone to convergence than expected. Furthermore, the sampling of these molecular data tend to imply that members of this *Cudonia/Spathularia* and Rhytismatales clade may be united by synapomorphic paraphysoid characters; namely, tendency toward paraphyses with curled or curved apices.

The basal positions of both the Neolectales in the Ascomycota and the Pezizales in the Euascomycetes results in the inference of the apothecium as being a primitive ascomal character state. However, due to the diversity of apothecial morphologies, the characterization of all apothecial types as representing primitive traits is probably somewhat inaccurate. Rather, the primitive ascomal trait of the Euascomycetes is best interpreted as possessing an exposed hymenium. Independent transitions from an exposed hymenium to enclosed hymenium resulted in the diversity of ascomata present in the Euascomycetes, *ie.*, peritheica, cleisothecia and pseudothecia. The analyses all also

corroborate hypotheses of paraphyses as synapomorphic for the Euascomycetes. Although *Neolecta* produces an ascoma similar in gross morphology to the earth tongues of the Helotiales, it does not form paraphyses. Rather, paraphyses appear to have been derived in the common ancestor of the Euascomycete, probably after the advent of ascomata. Importantly, if the placement of *Neolecta* outside of the Euascomycetes is accurate, then either the formation of ascogenous hyphae and ascoma arose twice – once in *Neolecta* and once in the ancestor of the Euascomycetes – or they arose once early in the evolutionary history of the Ascomycota and are symplesiomorphic for the Euascomycetes.

Phylogenetic hypotheses regarding the evolution of the lichen symbiosis are not resolved with these data. Additional taxon sampling in the combined nuclear SSU and LSU rDNA data set and the RPB2 data set may help clarify questions regarding the number of lichenization events within the euascomycetes and better polarize the character of lichenization. In addition, building on the principle of congruence from independent data sources, and utilizing a total evidence approach incorporating molecular data from more loci and more taxa may help us approach an organismal phylogeny and infer robust phylogenetic hypotheses for the euascomycete radiation.

The independent phylogenies presented here, corroborate inferred evolutionary trends and strengthen several hypotheses based on SSU rDNA data alone. The strong reciprocal corroboration of these independent data sets suggests that several phylogenetic inferences are robust and a more accurate phylogeny is emerging for some lineages of euascomycetes (De Queiroz et al., 1995; Hillis, 1995; Miyamoto and Fitch, 1995). The phylogenetic hypotheses from these loci seem to converge on several themes central to

traditional and modern ascomycete systematics. These include i) the polyphyly of the inoperculate discomycetes, ii) multiple convergences to the earth tongue morphology, iii) the primitive nature of the discomycete ascoma among the Euascomycetes, and iv) that paraphyses, but not necessarily ascomata, are synapomorphic for the Euascomycetes.

6.6 Acknowledgements

Several people derserve thanks for contributing unpublished sequences to this study. David S. Gernandt was particularly generous in providing a large number of SSU rDNA sequences representing members of the Rhytismatales and Helotiales. Joseph Spatafora, Francois Lutzoni, Arne Holst-Jensen, Yajuan Liu, and Jacqui Johnson also contributed significant sequence data. A Mycological Society of America Graduate Fellowship also supported a portion of this research.

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CHAPTER 7

Conclusion:

Strengthening Phylogenetic Hypotheses for Inoperculate Discomycetes and the Evolutionary History of Euascomycetes

Jamie L. Platt

7.1 Abstract

Phylogenetic hypotheses for the inoperculate ascomycetes have been tested and refined through cladistic analyses of sequence data from nuclear SSU rDNA, LSU rDNA, and *RPB2*, the gene encoding a subunit of RNA polymerase II. Phylogenies inferred from nuclear SSU rDNA data form the basis for many of the hypotheses tested because these characters are available for a broad range of taxa. Data from *RPB2* was used to test the robustness and potential accuracy of certain phylogenetic hypotheses. Independent gene phylogenies which infer similar phylogenetic relationship have been used as corroborative evidence that certain relationships do reflect the organismal phylogeny of the taxa involved. These inferred relationships that are well-supported and/or show correspondence in the independent gene phylogenies have been outlined in the preceeding chapters. Certain relationships remain unresolved however, and additional taxon and character sampling are needed. One problem remaining is the ordinal

placement of the Icmadophilaceae. Resolving this problem will involve a taxonomic redelimitation of the Helotiales which has been shown in these studies to be polyphyletic.

7.2 Introduction

The unsteady nature of traditional classifications for the inoperculate discomycetes prompting reference to the "unweildy Helotiaceae" and "nebulous borderland" between families (Maas Geesteranus, 1966) have been compounded by broadly overlapping morphological characters. In addition, these characters are subject to convergent evolution and may represent symplesiomorphies for a large heterogenous assemblage of taxa rather than synapomorphies and cladogenic events. On the other hand, morphological characters are important because they provide a practical means of categorizing morpho-groups and identifying taxa. Current issues of assessing biodiversity, both globally and locally, point to the utility of morphological characters for use in identification. From a systematic viewpoint however, understanding the phylogenetic history of these organisms allows us to test and refine hypotheses regarding evolutionary patterns which potentially provide even more information. Nucleotide and amino acid sequences from appropriate genes are ideal phylogenetic data from the standpoint that a relatively large number of multistate characters are available compared to morphological data. Problems of paralogous, rather than orthologous genes, notwithstanding, sequence data allows us to examine the evolution of lineages (phylogenies) through heritable traits (*i.e.*, genes).

The set of studies presented here has demonstrated the utility and power of molecular phylogenetic inference to test evolutionary hypotheses, improve classification

concepts, uncover patterns of convergent evolution, and point to potentially useful and phylogenetically meaningful morphological characters. However, this study has also pointed to some of the issues which must be addressed in order to construct robust and accurate molecular phylogenetic hypotheses. A brief summary of the information recovered in each portion of this dissertation research is outlined below.

7.3 Discussion

Because the Helotiales are primarily non-lichenized, the two families of lichens classified in the order, Baeomycetaceae and Icmadophilaceae (Hawksworth et al., 1995) appeared to offer an opportunity to examine a potentially recent transition to this symbiotic state and thereby polarize the lichen character. However, recent taxonomic changes also needed to be addressed. Namely the generic segregation of two common baeomycetoid lichens, *Baeomyces* and *Dibaeis*, was tested with phylogenetic analyses of nuclear rDNA data. This study inferred a phylogenetic history consistent with patterns of convergent evolution toward this baeomycetoid morphology. *Baeomyces* and *Dibaeis* were placed in two distinct, relatively disparate lineages (Chapter 2).

Another pattern of convergence was subsequently revealed with additional taxon sampling surrounding these lichenized lineages. The nonsexual lichens *Siphula* and *Thamnolia* were shown to be members of the Icmadophilaceae (Chapter 3). The terminal branches of these phylogenies are reflective of many of the teleomorph-anamorph patterns emerging with molecular phylogenetic data and the applicability of the anamorph concept in lichen systematics was introduced. The observation that many Ascomycete lineages appear to have lost sexual reproduction, may reveal a convergence

toward this reproductive character state, even in those lineages that have adopted a lichenized lifestyle. It was during this study that a fungal sequence with intragenic spatial phylogenetic variation (SPV) was detected (Chapter 4). The source of this variation is not easily assessed however, the effects that this type of data have on maximum parsimony analyses were striking and prompted a more thorough study of intragenic SPV and it's effect on maximum parsimony analyses.

The phylogenetic relationships among the non-lichenized members of the inoperculate discomycetes were also tested with molecular data. Nuclear rDNA was used to construct phylogenetic hypotheses for the earth tongues which were inferred to represent distinct lineages that independently converged upon the earth tongue ascomal morphology. The enigmatic affinity for some of these earth tongues (*Cudonia* and *Spathularia*) with endophytes of the Rhytismatales prompted additional sampling to obtain corroborative evidence from congruent independent, gene phylogenies. The large taxon sampling of SSU rDNA, the additional character sampling of combined SSU and LSU rDNA, and the independent protein-coding RPB2 gene all infer a close phylogenetic relationship between some earth tongues and endophytes and some common trends of convergence on ascomal morphologies.

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